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Monitoring levels of ascorbic acid derivatives in Sauvignon blanc (*Vitis vinifera* L.) during berry development and in the wine

by

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DECLARATION

I, **Mvuselelo Dalicuba**, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

M. Dalicuba	15 October 2020
Signed	Date

ABSTRACT

The exposure of plants to unfavourable conditions such as drought, high or low temperatures, heavy metals, salt stress and/or pathogenic attack increases the production of reactive oxygen species (ROS), thus inducing oxidative stress. One of the most important non-enzymatic antioxidants is ascorbic acid, which is used by plants to protect themselves against these toxic oxygen intermediates. The biologically active intermediate of ascorbic acid metabolism, dehydroascorbic acid (DHA), have antioxidant properties of its own. Diketogulonic acid (DKG) can also act as an antioxidant. DHA, DKG and L-L-threonate are ascorbic acid derivatives that are found during ascorbic acid catabolism. Catabolism refers to the breakdown of ascorbic acid when exposed to oxygen, especially in grape juice/wine.

The specific aims of the study were (i) to monitor and investigate the level of ascorbic acid derivatives in Sauvignon blanc (*Vitis vinifera* L.) during berry development until harvest; (ii) to monitor and investigate the effect of canopy management practices in the synthesis of ascorbic acid derivatives; (iii) to determine whether the trends in the development of ascorbic acid derivatives are the same for cooler and warmer areas; and (iv) to determine the influence of ascorbic acid on the pinking phenomena associated with Sauvignon blanc. Grape berries were sampled at different phenological stages according to the Eichorn-Lorenz scale (E-L 32 to E-L 38) from two grape-producing regions for the analysis of ascorbic acid and its derivatives DHA, DKG and L-threonate, with each region having north–south and east–west row orientations. Ascorbic acid derivative concentrations between the farms varied significantly. Both farms had high concentrations of L-threonate in all stages of ripeness, of the row direction. The DHA, DKG and L-threonate for Elgin were the highest at E-L 32 and the lowest for Wellington. No significant effect was observed for ascorbic acid derivatives between the row orientation comparisons on each farm block concerning the DHA, DKG or L-threonate content of the grapes. Ripening grape parameters such as pH, titratable acidity and sugar content of the berries were also measured at different grape ripening phenological stages on both farms. These parameters did not show any significant differences within the farm on the different vineyard row orientations ($p \geq 0.05$), but there were significant differences between the farms ($p \leq 0.05$) on different regions. Significant differences in pinking potential between the two climatic regions were observed in this study, with Elgin wines more susceptible to pinking and Wellington less susceptible. The study also added to the current knowledge that grapes grown in cool climates are more susceptible to pinking than grapes grown in warm climates.

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DEDICATION

This thesis is dedicated to my mother, Nowayini Dalicuba, and father, Msabalala Dalicuba, and my lovely, supportive sister, Lindeka Veronica Dalicuba; and everyone who supported me towards the completion of the thesis. Last but not least, I would like to thank the Mighty Lord for his presence in my life; without Him, I would be nothing.

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GLOSSARY

Asc	ascorbic acid
SO_2	sulphur dioxide
N_2	nitrogen
CO_2	carbon dioxide
O_2	oxygen
DHA	dehydroascorbic acid
DKG	2-3-diketogulonic acid
MP	methoxypyrazines
ETMP	3-ethyl-2-methoxypyrazine
SBMP	3-sec-butyl-2-methoxypyrazine
IPMP	3-isopropyl-2-methoxypyrazine
IBMP	3-isobutyl-2-methoxypyrazine
ROS	reactive oxygen species
TA	titratable acidity
MA	malic acid
C_2	carbon two
C_3	carbon three
C_4	carbon four
OxA	oxalic acid
TTA	total titratable acidity
TCA	trichloroanisole
LA	lactic acid
RDA	required dietary allowance
EAR	estimated average requirement
PVPP	polyvinylpolypyrrolidone
SAWIS	South African Wine Industry Information Service
SDLP	strand double lengthened Perold system
N–S	north to south
E–W	east to west
HPLC	high-performance liquid chromatography
H_2O_2	hydrogen peroxide
ARC	Agricultural Research Council
DAP	diammonium phosphate
FSO_2	free sulphur dioxide
TSO_2	total sulphur dioxide

PCA	principal component analysis
ANOVA	analysis of variance
RSA	South Africa

CHAPTER 1:
GENERAL INTRODUCTION AND BACKGROUND

1.1 Introduction

The exposure of grapevines to unfavourable environmental weather conditions such as drought, high or low temperature, heavy metals, chilling, freezing, salt stress and pathogenic attack increase the production of reactive oxygen species (ROS), thus inducing oxidative stress (Barata-Soares *et al.,* 2004; Krasensky & Jonak, 2012). One of the most important non-enzymatic antioxidants is ascorbic acid used by plants to protect themselves against these toxic oxygen intermediates (Barata-Soares *et al*., 2004). Ascorbic acid is an important redox cofactor in plant systems (known as a reducing agent) which carries out mono- and dioxygenase reactions by free-radical-dependent mechanisms (Steinberg & Rucker, 2013).

Ascorbic acid, also known as vitamin C, is defined as the generic term for all compounds exhibiting the biological activity of L-ascorbic acid (Lee & Kader, 2000). L-ascorbic acid was first isolated by Svirbely and Szent-Györgyi (1932), and its structure was established in 1933 (Iqbal *et al.,* 2004).

Ascorbic acid is naturally present in grapes, but the levels are low (below 10 mg/L) (Cojocaru & Antoce, 2015). Additions of ascorbic acid to wine were first allowed in the USA in 1957 (Cojocaru & Antoce, 2015). Ascorbic acid is commonly used as an antioxidant and to prevent pinking in certain grape must and wine (Bradshaw *et al.,* 2004), and has also been shown to have beneficially contributed to wine colour and flavour when added at bottling (Skouroumounis *et al*., 2005a). Moreover, the importance of ascorbic acid is realised in white juice of aromatic varieties in which the preservation of the aromatics is essential for wine quality, achieved through the addition of an ascorbic acid and sulphur dioxide (SO_2) combination, which acts as an aggressive antioxidant treatment (Skouroumounis *et al*., 2005b). Scientific researches has been done to understand the ripening berry parameters. These scientific efforts have been made to understand the complex series of physical and biochemical changes of grape berries during their development (Coombe, 1992; Zenoni *et al*., 2010 & Ali *et al.,* 2011). Today, however, the major concerns of viticulturists are berry size, colouration, monitoring ripeness, pH, acidity, and the volatile and non-volatile contents of the grape berry (Ali *et al.,* 2011). A view that seems to be generally accepted is that in some way, L-ascorbic acid is merely a by-product of plant metabolism; therefore it is evident that tartaric acid in grapes has L-ascorbic acid as a major precursor (Davies *et al.,* 2007). By far, the predominant acids are tartaric and malic acid, which together may account for over 90% of the total acidity in the berry, and which contribute the greatest to the pH of the juice, must and wine during vinification and subsequent ageing of wines (Conde *et al*., 2007). Ascorbic acid is a vital antioxidant compound that plays a critical role in the cellular metabolism of plants and animals, and

also pivotal to the production of collagen and is important in wound healing (Cruz-Rus *et al.,* 2010). The introduction of ascorbic acid as an intermediate in the reaction between oxygen and SO_2 in wine was considered to be beneficial. Oxygen could be scavenged quickly by ascorbic acid. The by-product of this reaction could then be mopped up by SO_2 present in the wine and most researchers recommended that ascorbic acid be used in conjunction with sulphur dioxide, not as a replacement. It was considered that this gave fresher wines with more fruit character.

The widespread use of SO_2 has led to some criticism of wine as a lifestyle beverage as SO_2 has been linked to severe allergy-like reactions in some asthmatics (Sapers, 1993; Zoecklein *et al.,* 1995). While there is no known replacement for the antimicrobial role of sulphur dioxide, it was suggested that ascorbic acid and its optical isomer, erythorbic acid, had the potential to replace SO_2 as the antioxidant in white wine (Bauernfeind & Pinkert, 1970; Sapers, 1993). However, complete replacement of SO_2 by ascorbic acid in white wine is not recommended as the aerobic oxidation of ascorbic acid produces hydrogen peroxide, considered to be a significant oxidant in wine (Zoecklein *et al*., 1995; Bradshaw *et al.,* 2002). Instead, ascorbic acid is used as a complement to SO_2 as both molecular SO_2 and hydrogen sulphite, the two active forms of SO_2 at wine pH, are capable of quenching hydrogen peroxide (Zoecklein *et al*., 1995; Peng *et al.,* 1998). The combined use of ascorbic acid and SO_2, therefore, requires considerable care as oxidation of ascorbic acid leads to a loss of SO_2 that, in turn, leads to a reduction in the main anti-microbial component (Barril *et al.,* 2012).

1.2 Problem statement

Grapevines (*Vitis* spp.) is one of the most economically important and widely cultivated fruit crops across the world (Vivier & Pretorius, 2002). The total arable land under grape production is approximately 7.4 million hectares with a production of 77.8 million tonnes in 2018, and 292 million hectolitres of wine (OIV, 2019). The grape berry is used as fresh or dried fruit, for the extraction of its juice, and most importantly, in wine production (Ali *et al.,* 2011). Ascorbic acid is rapidly synthesized during seed germination and continues to be produced in regions of active growth throughout the life of the grapevine (Loewus, 1999). In grapevines, ascorbic acid acts as a major cellular antioxidant protecting tissues against damage caused by reactive oxygen species, and it also serves as an enzyme co-factor (Smirnoff, 2001).

The biochemistry of ascorbic acid in plants is poorly understood (Davies *et al*., 2007), and according to the latest findings, there is insufficient information on the ascorbic acid concentrations in the grape berry during ripening and the resulting wine (Andrea-Silva *et al.*, 2014). Ascorbic acid, although naturally present in grapes, rapidly disappears throughout the ripening process and with exposure to oxygen during the crushing of grapes in the winemaking process (Iqbal *et al*., 2004). In addition to the importance of ascorbic acid in winemaking, ascorbic acid is vital for human growth

and maintenance of healthy bones, teeth, gums, ligaments and blood vessels as it acts as an antioxidant that chemically binds and neutralises the tissue-damaging effects of free radicals (Iqbal *et al*., 2004). L-ascorbic acid is found all over the plant world, often in significantly large quantities and distributed throughout the plant (Davey *et al*., 2000). The grape berry is, however, not among those fruit with a high ascorbic acid content, with approximately 11 mg per 100g fresh weight; as compared to the 200 mg per 100g fresh weight for blackcurrants and 50 mg per 100g fresh weight for lemons (Davey *et al*., 2000). Ascorbic acid is a precursor for the synthesis of tartaric and oxalic acids (Aziz & Sulaimani, 2013).

Knowledge of ascorbic acid in grape berries under South African (RSA) growing conditions is limited and inconclusive. While there is increasing interest in ascorbic acid accumulation in grape berries, little is known about the effect of terroir and vineyard practices on the concentration of ascorbic acid derivatives. Considering the importance of ascorbic acid on wine quality, the concentrations in grapes during berry development and in the resulting wine warrants further research. Consequently, optimal climatic conditions, the best climate, viticultural practices and post-harvest grape handling processes in maintaining high levels of ascorbic acid will be beneficial to make distinctive decisions on ascorbic acid addition in combination with SO_2. This study, therefore, seeks to investigate and monitor the impact of terroir and viticultural practices on ascorbic acid derivatives.

1.3 Ascorbic acid derivatives

Ascorbic acid derivatives were being monitored throughout grape berry ripening in Sauvignon blanc grapes in a cool and warm climatic conditions. Wines were made from these grapes to evaluate the presence of ascorbic acid derivatives in the final product. To our knowledge this is the first study of its kind in South Africa. The possible link between ascorbic acid concentration and the potential of the pinking phenomenon of Sauvignon blanc was also investigated. According to Simpson (1977), the increased pinking phenomenon in some vintages coincided with an increase in the use of colder fermentation and inert gas such as nitrogen (N_2) and carbon dioxide (CO_2), and closed containers to protect against oxidation. Wines produced under these conditions are often those exhibiting higher susceptibility to pinking when exposed to small amounts of air, often during bottling, and will become brown when further exposed to oxygen (O_2) at a later stage or when bottled with high dissolved oxygen content. This is in agreement with observations made by Singleton (1972). Examples of cultivars with higher potential to pink include Sauvignon blanc, Albarino, Riesling and Verdejo. Possible compounds responsible for pinking have not been identified, but are perceived to be phenolic compounds (Du Toit *et al.,* 2006; Andrea-Silva *et al*., 2014).

Ascorbic acid and the derivative levels were monitored throughout the grape berry ripening. The modified Eichorn-Lorenz (E-L) system from Coombe (1995) was used to monitor the ascorbic acid derivatives from EL stage 32 (pea-size) until stage 38 (harvest). The study also monitored the ascorbic acid derivatives, i.e. dehydroascorbic acid (DHA), 2,3-diketogulonic acid (DKG) and L-threonate levels from berry formation developmental stages E-L 32 (pea-sized) to E-L 38 (harvest). E-L refers to the modified Eichhorn and Lorenz developmental scale, as described by Coombe (1995). This research will aid winemakers to make distinct decisions on ascorbic acid management during winemaking, as it is added as an antioxidant agent. Too much ascorbic acid can result in browning of white wines, especially when insufficient SO_2 levels are used. This study also looks at the pinking potential of Sauvignon blanc from two wine-growing regions, and whether an ascorbic acid deficiency in wines causes pinking or not.

1.4 Aim and objectives

1.4.1 Aim

The specific aim of the study was to monitor the levels of ascorbic acid derivatives in Sauvignon blanc (*Vitis vinifera* L.) during berry development and in wine in South Africa.

1.4.2 Objectives

I. To determine the level of ascorbic acid derivatives in Sauvignon blanc (*Vitis vinifera* L.) during berry development until harvest on both farms, North-South and East-West row directions;

II. To determine the effect of canopy management practices in the ascorbic acid derivatives synthesis

III. To determine whether the trends in the development of ascorbic acid derivatives are the same for cooler and warmer areas;

IV. To determine the influence of ascorbic acid on the pinking phenomena associated with Sauvignon blanc Sauvignon blanc wines. As it is currently it is not clear where the pinking occurs. In the grapes or wine?

1.5 Research questions

I. Does the concentration of ascorbic acid derivatives differ during berry development?

II. Does canopy management practices influence ascorbic acid concentration?

III. Is there a difference in the trends in the concentrations of ascorbic acid derivatives between warm and cool climates?

IV. Does the concentration of ascorbic acid derivatives in juice and wine influence the pinking phenomenon?

1.6 Hypothesis

The hypothesis of the study was that:

I. The quantitative differences in ascorbic acid derivatives exist between different regions.
II. Vineyard rows that are non-suckered (i.e. more dense canopies) will have more ascorbic acid derivatives than the suckered vines.
III. Ascorbic acid derivatives of the warm and cool climates will depend on the factors affecting ascorbic acid synthesis in each region.
IV. Wines that have significantly more ascorbic acid will be better protected against pinking and oxidation than wines with less ascorbic acid.

1.7 Chapter outline

This thesis consists of five chapters. Chapter one provides the general introduction and background information of the study, problem statement, significance of the study and an outline of the study objectives, research questions and hypothesis. Chapter two gives a summary and analysis of the relevant literature on this topic. Chapter three provides a discussion of the material and methods that have been used in the study. Chapter four provides the results and discussion. Chapter five is the conclusion and recommendations of the study.

CHAPTER 2:
LITERATURE REVIEW

2.1 Introduction and background to Sauvignon blanc

Sauvignon blanc has been cultivated in France since the 18th century (Goussard, 2008). Planting of Sauvignon blanc has increased worldwide since 1960, and more recently at incredible rates in locations such as Australia, California, Chile, France, the eastern European countries, northern Italy and South Africa (Goussard, 2008). South African Sauvignon blanc wines are associated with a floral bouquet, green pepper, tomato leaf, box tree, blackcurrant bud, grapefruit, a delicate grassy taste and sought-after balance of fruit acid (Darriet *et al.,* 1995; Goussard, 2008). The cultivar characteristics include moderate to strong vigour, an upright growth habit and growing well on medium potential soils in cooler climates (Goussard, 2008; Robinson *et al.,* 2012). Bunches are very compact when the vines are grown on fertile soil and moderately susceptible to oidium and downy mildew, but because of its compact bunches, it is very susceptible to botrytis rot (Goussard, 2008; Robinson *et al*., 2012).

2.2 Sauvignon blanc sensory characteristics

Different cultivars are known to have different sensory characteristics, and one of the sensory modalities through which these differences are perceived is the sense of smell (Wilson, 2017). Wine aroma is an important part of wine appreciation as one of the intrinsic factors consumers use to judge wine quality (Coetzee & Du Toit, 2015). The human perception of wine aroma results from the mixture of volatile compounds in the wine and is affected by interactions between the volatile and the non-volatile components of the wine matrix (Wilson, 2017). These volatile aroma compounds can drive differences between varieties and styles of wine, allowing for volatile "fingerprinting" and identification of impact compounds (Fischer, 2007; Polášková *et al.*, 2008).

The Sauvignon blanc wine aromatic profile can generally be attributed to three distinct and very potent groups of compounds; thiols, esters and methoxypyrazines (MP) (Šuklje *et al.,* 2016). The 3-sulfanylhexan-1-ol (3SH) and 3-sulfanylhexyl acetate (3SHA) in Sauvignon blanc wines are often associated with tropical fruit aromas such as passion fruit, mango, guava, gooseberry, grapefruit and have a detection threshold as low as 60 and 4 ng/L, respectively, in model wine solutions (Coetzee & Du Toit, 2012). The methoxypyrazines, namely 3-isobutyl-2-methoxypyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP), are responsible for the green, vegetative, green pepper and asparagus aromas of Sauvignon blanc wines (Allen *et al.,* 1991). The detection threshold of IBMP in water and white wine is as low as 2 ng/L (Allen *et al.,* 1991) and its concentrations in wines are strongly correlated with those observed in the corresponding grapes

(Šuklje *et al.,* 2014). The main MP found in grapes, musts and wines are 3-ethyl-2-methoxypyrazine (ETMP), 2-sec-butyl-2-methoxypyrazine (SBMP), 3-isopropyl-2-methoxypyrazine (IPMP), and 3-isobutyl-2-methoxypyrazine (IBMP) (Mozzon *et al.,* 2016). The nature of the alkyl radical largely determines the aromatic perceptions of these compounds (Sala *et al.,* 2004), as summarised in Table 2.1.

Table 2.1: Odour descriptors and olfactory threshold in water (ng/L) of the main alkyl methoxypyrazines (MP) according to Sala *et al* (2004)

Alkyl methoxypyrazines	Odour description	Threshold (ng/L)
ETMP	Raw potato, earthy, green, bell pepper	400-425
SBMP	Green, ivy leaves, bell pepper	1-2
IPMP	Earthy, cooked asparagus, green pepper	2
IBMP	Earthy, green, bell pepper, musty	0.5-2

ETMP: 3-ethyl-2-methoxypyrazine, SBMP: 3-sec-butyl-2-methoxypyrazine, IPMP: 3-isopropyl-2-methoxypyrazine, IBMP: 3-isobutyl-2-methoxypyrazine

IBMP is typically the most predominant MP in grape and wine and, as such, is a major contributor to the vegetative character (Sala *et al*., 2004). This vegetative character is most commonly, although not exclusively, associated with Sauvignon blanc, Cabernet Sauvignon and other varietals (Allen *et al*., 1991).

2.3 Berry development and ripening

2.3.1 Fruit ripening

Fruit ripening is a dynamic process involving visible changes in fruit morphology as well as changes in genetic and biochemical properties and are only revealed upon closer analysis (Coetzee & Du Toit, 2012). Fruit ripening is an oxidative process that is characterised by the release of reactive oxygen species such as hydrogen peroxide (Brennan & Fenkel, 1977). The production of reactive oxygen species (ROS) during phenological development or in response to an environmental stimulus is genetically programmed, and the cellular accumulation of ROS is regulated by the antioxidant system of the plant (Foyer & Noctor, 2005). During fruit ripening, many reactions such as colour transformation, sugar synthesis and cell wall degradation occur (Coetzee & Du Toit, 2012), and these phenomena may cause tissue stresses, which would require antioxidant action especially by ascorbate, preventing cell damage (Barata-Soares *et al*., 2004). It is conceivable that due to these stresses, ascorbic acid levels would invariably decrease during fruit ripening (Barata-Soares *et al*., 2004). However, it would appear that in some fruit like oranges, ascorbic acid levels increase greatly, while in others, these levels remain unchanged or decrease (Barata-Soares *et al*., 2004). Ascorbate is a major derivative in plants, as it acts as an antioxidant and in association with other components of the antioxidant system to protect plants against

oxidative damage resulting from aerobic metabolism, photosynthesis and a range of pollutants (Smirnoff, 1996). Ascorbic acid is best known for its function as an antioxidant and its role in collagen synthesis in humans (Smirnoff, 1996).

2.3.2 Stages of grape berry development

Berry growth consists of two successive sigmoid curve that are separated by a lag phase (Coombe & Iland, 2005) (Figure 2.1A&B). The first cycle (stage I) represents berry formation or cell division, and during the second cycle, the berry enlarges, or cells expand that culminates in ripening (Coombe & Iland, 2005).

During stage II (Figure 2.1A&B), berries actively accumulate organic acids and small quantities of sugar (Dokoozlian & Kliewer, 1996). At the end of the lag phase, véraison (a French word meaning the commencement of berry colour change) marks the beginning of ripening (Biondi, 2007). During véraison, the berries soften and the skin changes from green to purple in red-fruited cultivars, while in white-fruited cultivars the berries acquire a more translucent appearance (Biondi, 2007). The véraison process can continue for two weeks until all berries in the cluster complete the colour change (Harris *et al.,* 1968; Coombe & Iland, 2005). Water importation through the xylem declines gradually while the berry changes colour (Biondi, 2007). Although the xylem appears to remain functional (Keller *et al.,* 2006), most of the water for the berry is now provided through the phloem (Keller *et al.,* 2006). Berry turgor pressure is low (<0.5 bar), but remains positive and relatively constant throughout ripening (Matthews & Shackel, 2005).

Stage III (Figure 2.1A&B), represents slowing down after stage II, ending as ripening starts (Coombe & Iland, 2005). The second cycle (stage III) commences with berry softening, berry colouring and a renewed increase in size (Kanellis & Roubelakis-Angelakis, 1993; Coombe & McCarthy, 2000). Sugar and colour accumulate rapidly in the berry during stage III, with a sharp decline in the total organic acid concentration (Dokoozlian & Kliewer, 1996). The pattern of decline of titratable acidity (TA) corresponds with the sharp decline in the malic acid (MA) content (Crippen & Morrison, 1986). Accumulation patterns of malic and tartaric acids differ during the sigmoidal growth (Crippen & Morrison, 1986). Despite tartaric acid being intensively accumulated during rapid cell division following anthesis, malic acid is present in concentration two to three times higher before the start of ripening (Crippen & Morrison, 1986; Terrier & Romieu, 2001).

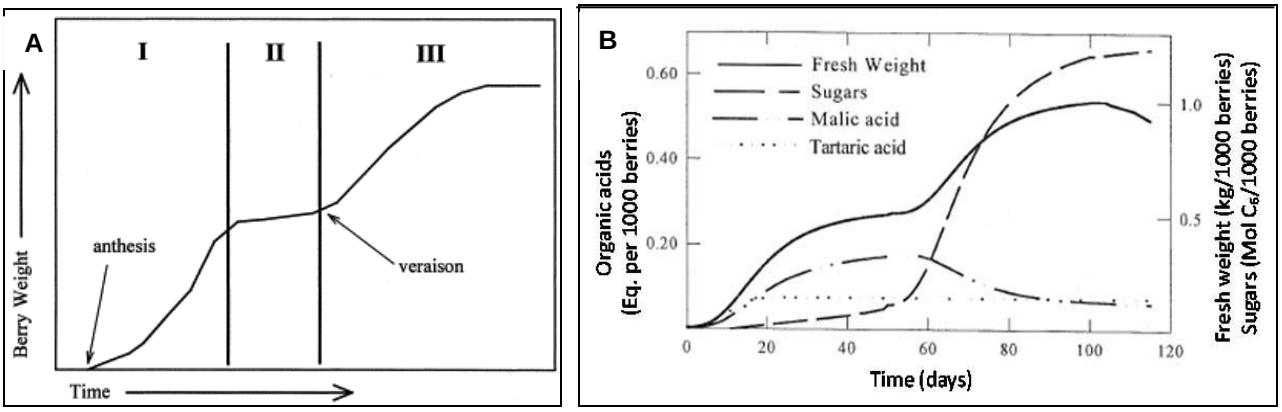

Figure 2.1 (A&B): The three major developmental stages from Coetzee (2013) master thesis

(Coombe 1973)

The three major developmental stages, as noted by Coombe (1973) to the left, illustrating the double sigmoidal curve of berry development (Figure 2.1A). The figure to the right illustrates the typical changes of the content of malic- and tartaric acid as well as sugar in the grape berry during development (Figure 2.1B). The change in fresh mass is also indicated (Terrier & Romieu, 2001)

2.3.3 Ascorbic acid contents of other fruits

Ascorbic acid contents of fruits and vegetables are also variable among cultivars and tissues (Lee & Kader, 2000). Nelson *et al.* (1972) found a range from 19.3 to 71.5 mg/100g ascorbic acid in six strawberry cultivars from four locations. Lee *et al.,* (1995) reported a range of 64 to 168 mg/100g ascorbic acid in five fresh bell pepper cultivars. In citrus fruit, flavedo (coloured outer peel layer of citrus fruits) tissue contained four times higher ascorbic acid content than the juice (Nagy, 1980).

Table 2.2: Ascorbic acid content (mg/100g fresh weight (f.w)) of some fruits

Commodity	Fresh/5-20 days storage	Ascorbic acid (mg/100g)	Dehydroascorbic acid (mg/100g)	References
Banana	Fresh	15.3	3.3	Vanderslice *et al*. (1990)
Blackberry	Fresh	18.0	3.0	Agar *et al*. (1997)
	20% CO_2, 9 days at 1°C	16.5	3.0	Agar *et al*. (1997)
Black current	Fresh	86.0	6.0	Agar *et al*. (1997)
	20% CO_2, 20 days at 1°C	61.0	3.0	Agar *et al*. (1997)
Cantaloupe	Fresh	31.3	3.0	Vanderslice *et al*. (1990)
Grapefruit	Fresh	21.3	2.3	Vanderslice *et al*. (1990)
Kiwifruit	Fresh	59.6	5.3	Agar *et al*. (1999)
	slices, 6 days at 10°C	39.4	12.1	Agar *et al*. (1999)
Lemon	Fresh	50.4	23.9	Mitchell *et al*. (1992)
Mandarins	Fresh	34.0	3.7	Mitchell *et al*. (1992)
Orange (California)	Fresh	75.0	8.2	Vanderslice *et al*. (1990)
Orange (Florida)	Fresh	54.7	8.3	Vanderslice *et al*. (1990)
Persimmon	Fresh	110.0	100.0	Wright & Kader (1997)
	12% CO_2, 5 days at 5°C	122.0	87.0	Wright & Kader (1997)
Raspberry	Fresh	27.0	2.0	Agar *et al.* (1997)
	(20%CO_2, 2% O_2, 9 days at 1°C	22.0	5.0	Agar *et al.* (1997)
Strawberry	Fresh	60.0	5.0	Agar *et al*. (1997)
	20% CO_2, 20 days at 1°C	27.0	34.0	Agar *et al*. (1997)

2.4 Synthesis of ascorbic acid in plants

2.4.1 Ascorbic acid synthetic pathways

Ascorbic acid (Figure 2.2) is a natural compound found in most fruits and vegetables (Du *et al.*, 2012). Plants and most animals synthesize ascorbate from glucose (Du *et al*., 2012). The last two decades have witnessed groundbreaking research progress in unravelling the biosynthesis of ascorbic acid in plants with several pathways suggested (Lorence *et al.,* 2004; Cruz-Rus *et al.,* 2010). Melino *et al.,* (2011) found that ascorbic acid accumulation and its catabolites coincide with the photosynthetic rate of *Vitis vinifera* berries and leaves. This can be attributed to the light dependency of ascorbic acid accumulation being linked to its functioning in the chloroplast, where it is found in varying concentrations (Coetzee, 2013).

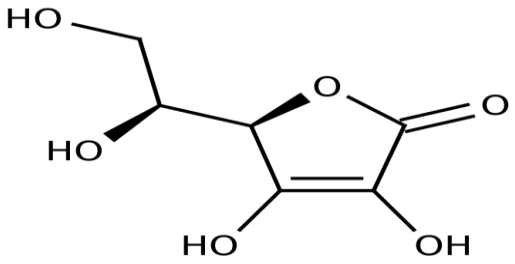

Figure 2.2: Ascorbic acid structure

Source: www.chemspider.com; accessed 02 March 2018)

The first pathway occurs in *Vitis vinifera* and experimentation showed that the primary step for the formation of total acidity was the cleavage of L-ascorbic acid between carbon four (C_4) and carbon five (C_5) and the C_4 fragment consequently converted to tartaric acid (Wagner *et al.,* 1975; Loewus, 1999) as can be seen in Figure 2.3. The residual two-carbon compound, glycolaldehyde (Conde *et al.*, 2007) is recycled back to a hexose phosphate (Williams *et al.,* 1979; Loewus, 1999).

In the second pathway, L-ascorbic acid is cleaved between carbon two (C_2) and carbon three (C_3), which yields oxalic acid and L-threonate and in turn converted in plants of the Geraniaceae to tartaric acid in the leaves (Loewus, 1999; DeBolt *et al.,* 2006). DeBolt *et al*. (2004) found that in whole berries of *Vitis vinifera,* both oxaloacetic acid and tartaric acid is formed through ascorbic acid catabolism, suggesting that in grapevines, both pathways are functional. Radio-isotope tracer studies revealed the direct pathway of the catabolism of ascorbic acid to tartaric acid. This is illustrated in Figure 2.3 (Coetzee, 2013). DeBolt, Melino and Ford (2007) and Kanellis and Roubelakis-Angelakis (1993) indicated that ascorbic acid is converted to 2-keto-L-idonic acid, successively reduced to L-idonic acid and oxidized to 5-keto-D-gluconic acid. Later literature substituted 2-keto-L-iodic acid with 2-keto-L-gluconic acid, as indicated in Figure 2.3 (Conde *et al.*, 2007). To date, the only enzyme isolated in the second pathway is L-idonate dehydrogenase, responsible for the conversion of L-idonic acid to 5-keto-D-gluconic acid as indicated in Figure 2.3 (DeBolt *et al.*, 2006; Martínez-Esteso *et al.*, 2011).

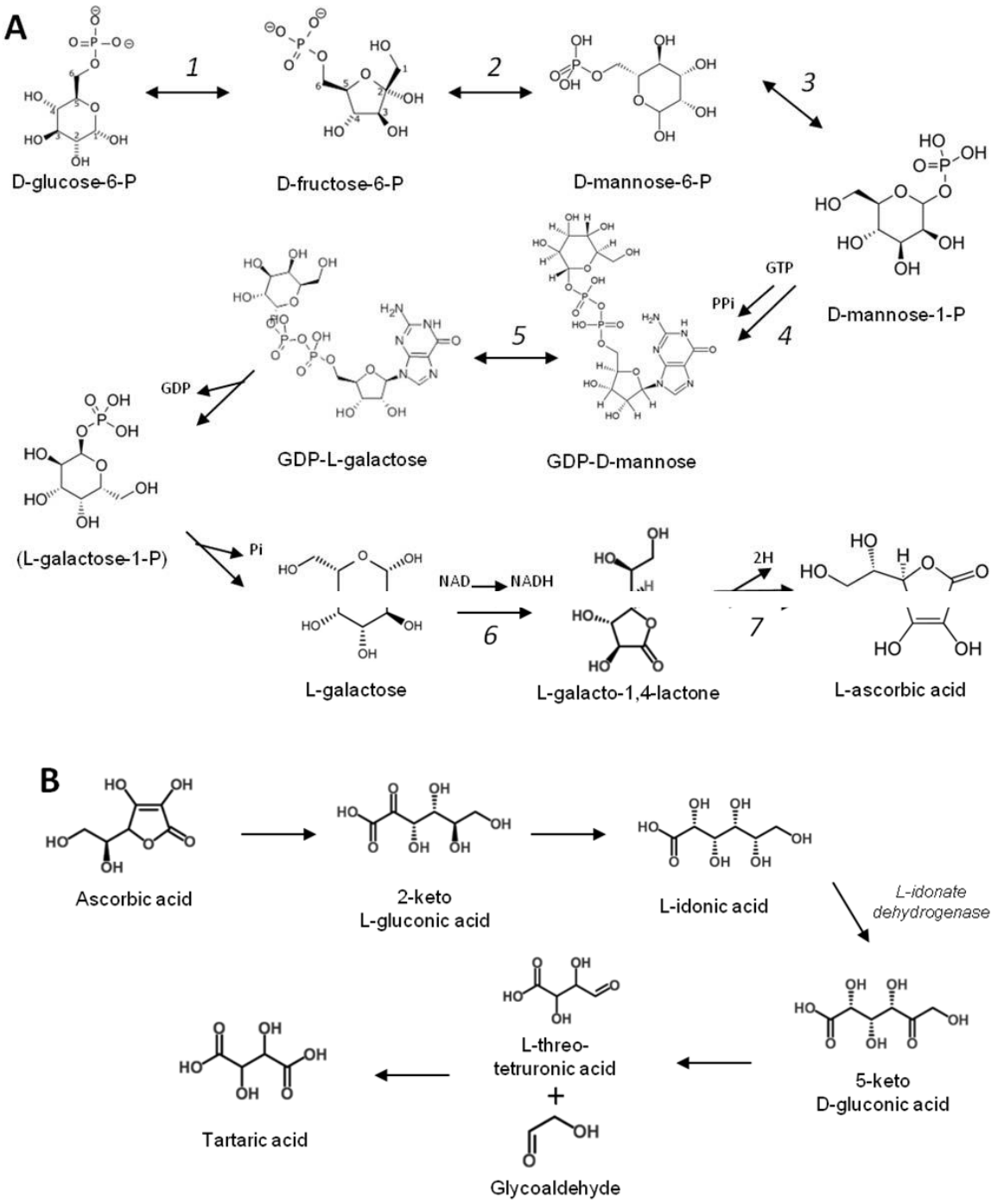

Figure 2.3: (**A) The Smirnoff–Wheeler pathway proposed for L-ascorbic acid biosynthesis in higher plants, (B) the proposed pathway for tartaric acid formation from ascorbic acid in the grapevine**

(Coetzee, 2013)

The Smirnoff-Wheeler (A) pathway proposes pathways for L-ascorbic acid biosynthesis in higher plants (Loewus, 1999). The enzymes identified in L-ascorbic acid biosynthesis are: (1) hexose phosphate isomerase; (2) phosphomannose isomerase; (3) phosphomannose mutase; (4) GDP-D-mannose pyrophosphorylase; (5) GDP-D-mannose-3,5-epimerase; (6) L-galactose dehydrogenase; and (7) L-galactono-1,4-lactone dehydrogenase (Coetzee, 2013). The proposed pathway for tartaric acid (Figure 2.3 B) formation from ascorbic acid in the grapevine (as adapted from Loewus, 1999 & DeBolt *et al.,* 2007) indicate the presence of L-idonate dehydrogenase (Conde *et al.*, 2007), which is also seen as the rate-limiting step in the pathway (DeBolt *et al.*, 2006). Reduction of the ascorbate breakdown product 2-keto-L-gulonic acid to L-idonic acid constitutes a critical step in this L-tartaric acid biosynthetic pathway (Jia *et al*., 2019).

2.4.2 Grape berry ascorbic acid contents

Grape berries do not accumulate large quantities of ascorbic acid compared to other fruits (Melino *et al.,* 2009b). For example, fruit that are particularly rich in ascorbic acid include blackcurrants (with 11.2–11.8 µmol/g fresh weight), strawberries (3.37 µmol/g fresh weight), kiwifruits (3.41 µmol/g fresh weight), cranberries (0.67 µmol/g fresh weight), apples (0.11–0.56 µmol/g fresh weight), and apricots (0.39–0.56 µmol/g fresh weight) (Melino *et al.,* 2009b). Melino *et al.* (2009b) demonstrated that Shiraz, at commercial ripeness, had accumulated ascorbic acid levels of 0.43–0.69 µmol/g fresh weight (see Figure 2.4). In some higher plants (trees, shrubs, flowering herbs), including *Vitis vinifera* L. (Loewus, 1999), the tartaric acid pathway is the result of L-ascorbic acid catabolism (DeBolt *et al.,* 2006) via the conversion of L-idonate to 5-keto-D-gluconate (Saito & Kasai, 1984) under the action of L-idonate dehydrogenase (Ruffner, 1982).

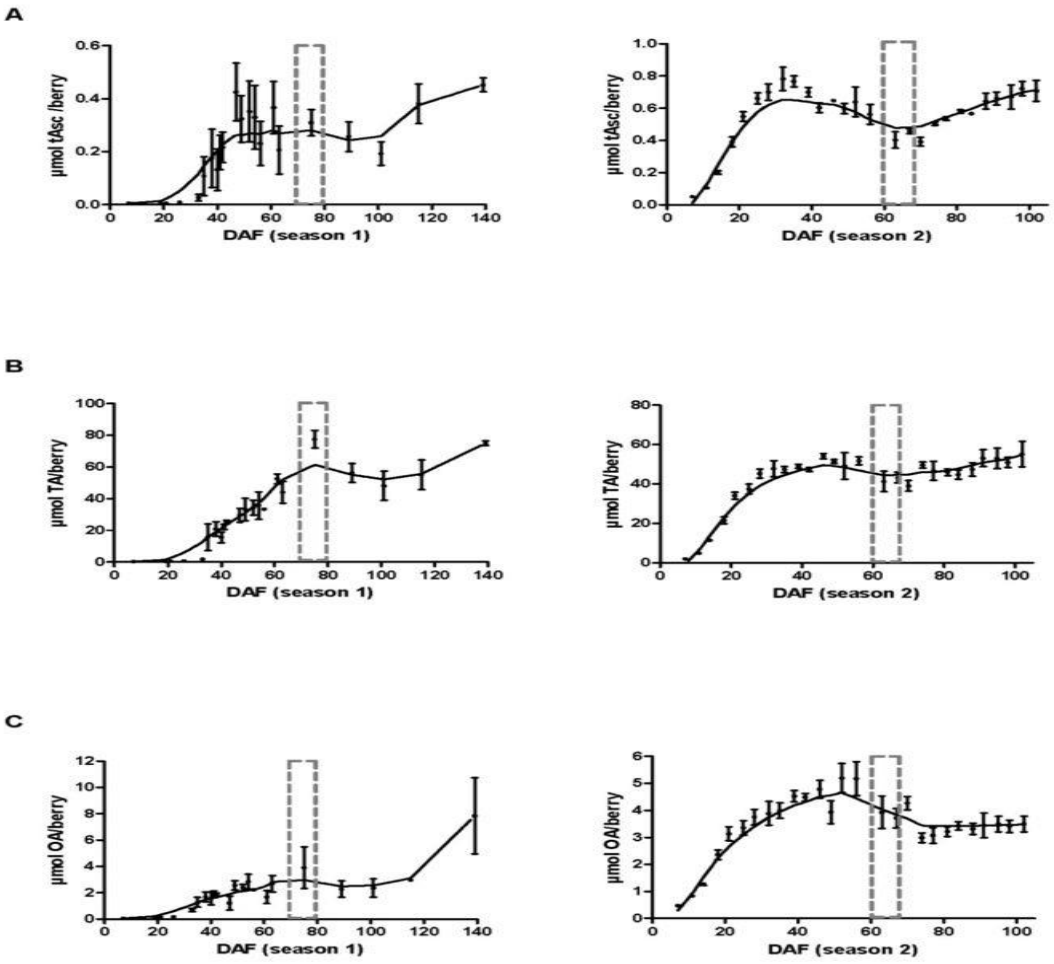

Figure 2.4: Accumulation of total ascorbic acid and the ascorbic acid catabolites tartaric acid and oxalic acids

(Melino *et al.,* 2009b).

All graphs in the left-hand panel show *Vitis vinifera* c.v. Shiraz berries grown between 2005 and 2006 (season 1) where n = 3 and the standard error of the mean is indicated. All graphs in the right-hand panel show *V. vinifera* c.v. Shiraz berries grown between 2007 and 2008 (season 2) where n = 4 and SEM is indicated. A shows the accumulation of total ascorbic acid, B shows the accumulation of tartaric acid, and C shows the accumulation of oxalic acid. The developmental stage of véraison is indicated by a grey dotted box (Melino *et al.,* 2009b)

Whereas the catabolic pathway for ascorbic acid in grapes has been partially described, very little is known about the synthesis of ascorbic acid in this fruit (DeBolt *et al*., 2006). The ascorbic acid content of the photosynthetic tissue is fairly comparable across species, whereas huge interspecies variability is observed in storage organs (Hancock *et al.,* 2003). Ascorbic acid accumulation is regulated by both biosynthesis and turnover *in vivo* and in some plants by translocation from photosynthetic tissue (Hancock *et al*., 2003).

2.4.3 Ascorbic acid derivatives

Oxidative degradation of white wines is a problem for winemakers worldwide and results in loss of characteristic aromas of young wines, namely their floral and fruity notes, and the development of brown colour and oxidised aroma (Skouroumounis *et al*., 2005a). Ascorbic acid oxidation is also a problem encountered by researchers during analysis of the ascorbic acid content of fruit (Davey *et al*., 2000; Melino *et al.,* 2009a). Ascorbic acid is stable when dry, but solutions readily oxidise, especially in the presence of trace amounts of copper, iron and alkali (Davey *et al*., 2000), and greatly favoured by the presence of oxygen, light, heavy metal ions, especially Cu^{2+}, Ag^{+}, and Fe^{3+}, and by alkaline pH, high temperature, and enzymes (Lee & Kader, 2000). Ascorbic acid extraction is made difficult by its unstable nature (DeBolt *et al*., 2006). As a water-soluble reducing agent and donor antioxidant, ascorbic acid can undergo two consecutive, one-electron oxidations resulting in the formation of ascorbate radical (Asc−) and dehydroascorbic acid (Figure 2.5) (Du *et al*., 2012).

2.4.3.1 Dehydroascorbic acid (DHA)

According to Cadenas (1995), the first oxidation product of ascorbic acid is the radical, monodehydroascorbate (MDHA), also known as semidehydroascorbate, or ascorbate free radical. DHA itself is unstable and undergoes irreversible hydrolytic ring cleavage to 2,3-diketogulonic acid in aqueous solution (Deutsch, 1998d). DHA can be reduced to ascorbic acid by reducing agents and also can be irreversibly oxidized to form diketogulonic acid, which has no ascorbic acid activity (Parviainen & Nyyssonen, 1992). The half-life for this breakdown was found to be six minutes at 37°C (Rose, 1987), although the rates of ascorbic acid oxidation and DHA hydrolysis will be influenced by factors such as concentration, temperature, light and pH (Davey *et al*., 2000).

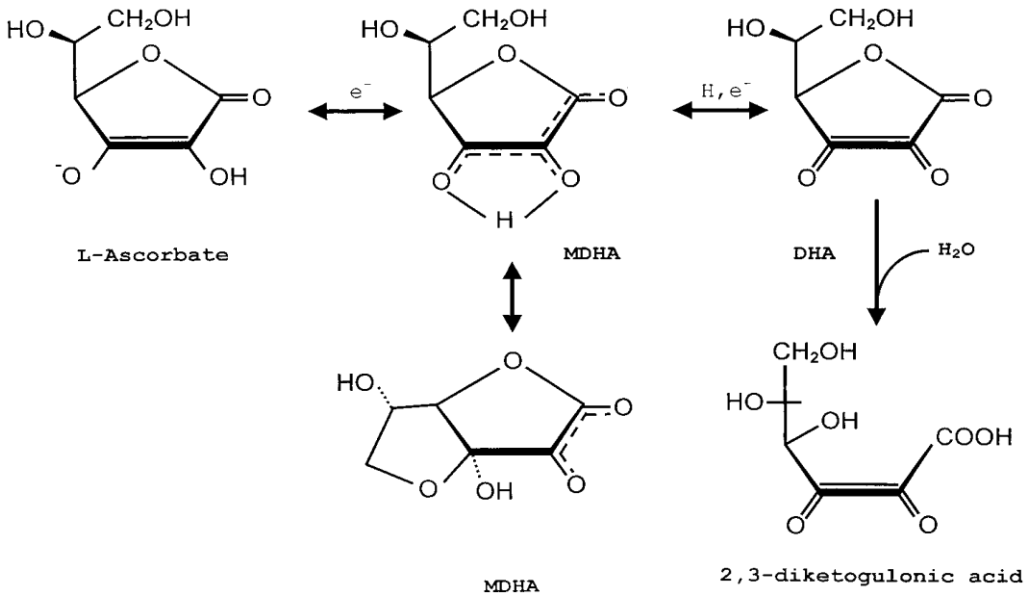

Figure 2.5: Oxidation of L-ascorbate

(Davey et al., 2000)

The final stable products of DHA catabolism differ between species, with oxalic- and threonic acid being the most common (Truffault *et al.,* 2017). According to Melino *et al*. (2009b), DHA can be enzymatically reduced to re-form ascorbic acid. DHA is unstable at physiological pH, and if not rapidly reduced to ascorbic acid or used in other synthetic processes, it is hydrolysed to 2,3-diketo-L-gulonic acid (Melino *et al*., 2009b). This step results in the loss of ascorbic acid *in vivo*, which is minimised by the existence of ascorbic acid recycling mechanisms (Davey *et al*., 2000). The low pH of the fruit stabilises ascorbic acid; however, post-harvest treatment such as storage and industrial processing can lead to a loss in ascorbic acid via oxidation (Davey *et al*., 2000). Sample preparation methods must, therefore, be designed to reduce the preparation time and minimise the oxidation of ascorbic acid (Hewitt & Dickes, 1961). The oxidation of ascorbic acid leads to the production of hydrogen peroxide, a well-known oxidant (Bradshaw *et al*., 2011) (Figure 2.6).

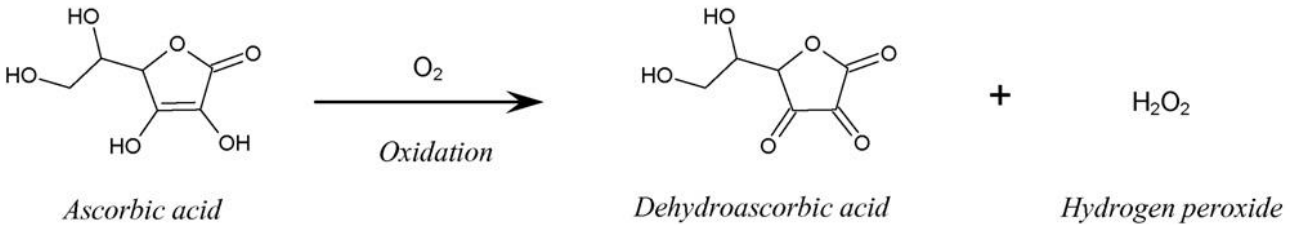

Figure 2.6: Mechanism of ascorbic acid oxidation

(Bradshaw et al., 2011)

2.4.3.2 Diketogulonic acid (DKG)

Under conditions of oxidative stress, DHA, after being hydrolysed to DKG, rapidly degrades to 5 and 4 carbon species (Deutsch, 2000). DKG is the degradation product of DHA (Nemet & Monnier, 2011). At physiological conditions, DHA rapidly hydrolyses to 2,3-diketogulonate (DKG), which is quite unstable and metabolises further to form L-threonate (Parsons & Fry, 2012). DKG itself can be oxidised into an unknown compound ('H'), which itself can be further oxidised to L-threonate; therefore, DKG can, like ascorbic acid and DHA, act as an antioxidant (Parsons *et al.,* 2011). L-threonate, on the other hand, is quite stable in an oxidative environment (Devamanoharan *et al.,* 1996).

2.4.3.3 Threonic acid

L-threonate can alternatively be produced from DKG degradation. Major oxidative end-products of irreversible DHA degradation, both *in vitro* and in most plant species *in vivo*, are L-threonate plus oxalic acid (OxA), which are formed by oxidative cleavage of the C-2–C-3 bond (Deutsch, 1998a). Figure 2.7 shows a series of intermediates between DHA and its end-products. It was proposed that the first-formed product of DHA oxidation was cyclic oxalyl L-threonate, whose two ester bonds were successively hydrolysed to produce first oxalyl l-L-threonate and finally oxalic acid and L-threonate (Green & Fry, 2005).

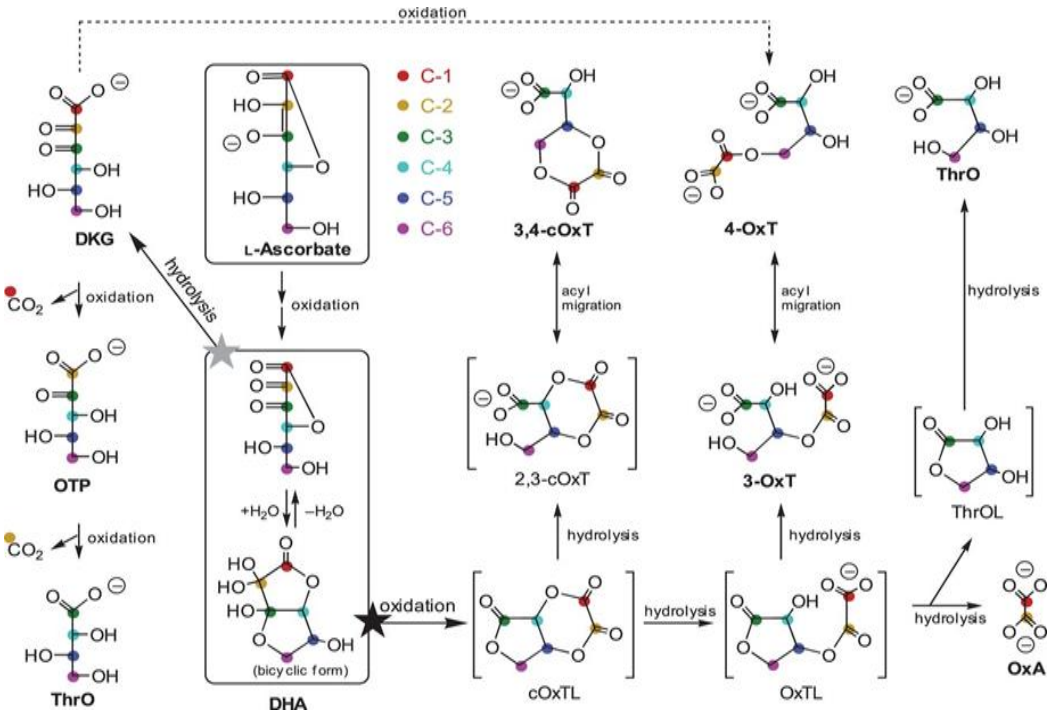

Figure 2.7: Proposed structures and interconversion of ascorbate derivatives

(Parsons & Fry, 2012)

Ascorbate is oxidised non-enzymically or by ascorbate oxidase to yield dehydroascorbic acid, which is shown both in its conventional structure and as the more realistic bicyclic structure (see Figure 2.7) (Parsons & Fry, 2012). DHA is further oxidised non-enzymically by H_2O_2 (and possibly enzymically *in vivo*), in a reaction that cleaves the bond marked '∗'. The product is a highly reactive intermediate proposed to be cyclic-2,3-*O*-oxalyl-l-threonolactone (cOxTL; centre structure), which is subject to any of the three hydrolytic reactions shown, selected stochastically (Parsons & Fry, 2012). If not rapidly oxidised, DHA can be hydrolysed to diketogulonate (DKG), which is shown only in its conventional structure, although this too is likely to be hydrated at the 'di-keto' group in aqueous solution (Parsons & Fry, 2012).

2.5 Other acids in grape berries

2.5.1 Organic acids

Acids are one of the major components influencing the sensory perception of wine and originate largely from the berry (Coombe & Iland, 2005; Coetzee, 2013). The presence of organic acids in adequate concentrations in the grape berry, of which tartaric and malic acid are the main organic acids present, is important as this determines the potential of a must to produce a good and stable wine (Boulton *et al.,* 1998; Coetzee, 2013). The organic acids present in grapevines are mainly intermediates of metabolic pathways operating in the grape berries (Kanellis & Roubelakis-Angelakis, 1993). Wine pH, an important indicator of grape quality, together with flavour and colour, indicates the strength of these acids as well as the presence of other ions that are present in the wine (Coetzee, 2013). The three main factors influencing wine pH include the ratio of malic to tartaric acid, the total amount of acid present in the wine and the quantity of potassium (K^+) (Conde *et al.*, 2007; Coetzee, 2013). Acids furthermore influence the physical, biochemical and microbial stability of the wine (Volschenk, Van Vuuren & Viljoen-Bloom, 2006). The concentration of acids and their corresponding salts vary according to grape variety, season, vine location, cultural conditions and the state of maturity of grapes (Watson, 2003).

There are at least 27 free organic acids present throughout the grapevine (Kliewer, 1966). The dicarboxylic acids, namely malic and tartaric acid, contribute from 68% to 92% of the total acids found in the berries and leaves (Jackson, 2008). In turn, the percentage contribution of malic and total acidity to the total juice volume is 0.2 to 1% and 0.3 to 1.5%, respectively (Winkler, 1962; Coetzee, 2013). Other organic acids present in the wine are amino acids (1 to 3 g/L) and several other to a lesser extent such as acetic, galacturonic, pyruvic and keto-glutaric acid (Boulton *et al*., 1998). The occurrence of this number of organic acids, including all the intermediates of glycolysis, the Krebs and glyoxylic acid cycles and the shikimic acid pathway, suggests that several metabolic cycles are involved (Coetzee, 2013). Malic acid and tartaric acid are secondary products of sugar metabolism, primarily synthesized in the berry and to a lesser extent in the leaves (Coetzee, 2013).

No conclusive evidence exists of transport of either of malic acid or tartaric acid from the leaves to the fruit (Watson, 2003; Coetzee, 2013).

In wine, organic acids quantitatively dominate the acid composition (Ribéreau-Gayon, 1968; Jackson, 2008), with tartaric acid and malic acid contributing to more than 90% of the total acids found in grape berries and wine (Winkler, 1962). Among all these acids, malic acid demonstrates the highest growth of its content during ripening of grapes (Jančářová *et al.,* 2013). Malic acid and tartaric acid are actively synthesized up until véraison (Ruffner, 1982). However, part of the tartaric acid is bound with potassium in the form of potassium hydrogen tartrate (Jančářová *et al*., 2013), after which the content of tartaric acid remains relatively stable in the berry (DeBolt *et al*., 2004) and the malic acid content decreases due to the metabolism of malic acid through different pathways (Sweetman *et al*., 2009; Jančářová *et al.,* 2013). Malic and tartaric acids also contain lower levels of succinic, oxalic and citric acids (Stratil*,* Kuban & Fojtov*,* 2008; Jančářová *et al.,* 2013). These acids are produced from different biosynthetic pathways (Coetzee, 2013). Tartaric acid is synthesized from ascorbic acid (DeBolt *et al*., 2007), with malic acid being an intermediate in grapevine metabolism (Ruffner, 1982). Tartaric acid and malic acid are secondary products of sugar metabolism and primarily synthesized in the berry (Watson, 2003). In the grape berry, both the tartaric and malic acid syntheses have been reported to occur until véraison, declining in the later stages (DeBolt *et al*., 2007). Additional possibilities responsible for the observed decrease in acid concentrations are noted by Ruffner, Brem and Malipiero (1982) and Kanellis & Roubelakis-Angelakis (1993) as the dilution in acid concentration as berry volume increases, the inhibition of acid synthesis coincides with an increase in malic acid degradation and the transformation of acid to sugar. The tartaric acid to malic acid ratio at harvest varies considerably according to the grape variety (Ruffner, 1982; Kanellis & Roubelakis-Angelakis, 1993).

2.5.2 Tartaric acid

Tartaric acid is economically the most important acid as it is the main organic acid controlling the juice pH and, therefore, in determining the suitability of the grapes for winemaking (Coetzee, 2013). Tartaric acid is found specifically in grapes, and it does not occur in other fruits in substantial amounts (Jančářová *et al.,* 2013). It is found in all parts of grape berries; grapevine leaves contain 13 000 to 16 000 mg/L of tartaric acid (Jančářová *et al*., 2013). Furthermore, through the addition of tartaric acid during vinification, oxidative and microbiological spoilage can be minimised, thus improving the organoleptic and ageing potential of the completed wine (DeBolt *et al.*, 2007). Tartaric acid, the stronger and more stable acid (Dry & Coombe, 2005), is the primary non-fermentable soluble acid in grapes and the principal acid in wine (Hale, 1962), contributing important aspects to the taste, mouthfeel, ageing potential of the vinified product, and the main

organic acid determining the suitability of the grapes for winemaking as it controls the juice pH (DeBolt *et al*., 2007).

Contrary to other cultivated fruit where malic acid and citric acid accumulation are more common, grapes accumulate significant quantities of tartaric acid, which remains in the berry during ripening (Watson, 2003; DeBolt *et al.*, 2007).

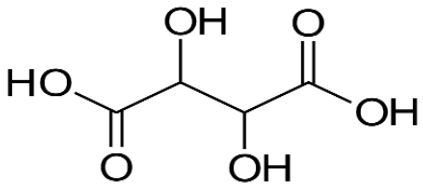

Figure 2.8: The chemical structure of L-tartaric acid

(www.chemspider.com; accessed 2 March 2018)

In the family Vitaceae, tartaric acid is present as an optically active L-(+)-stereoisomer (Wagner *et al.*, 1975). Contrary to most organic acids, the metabolic origin of tartaric acid is situated outside of the oxidative metabolism of sugars and originates with L-ascorbic acid (Conde *et al.*, 2007), which in turn is synthesized from glucose (Loewus, 1999). The biosynthesis of tartaric acid is limited to the period from post-anthesis up to véraison (Conde *et al.*, 2007). Thereafter, the concentration of tartaric acid decreases due to an increase in the berry volume, but the tartaric acid content in the berry remains relatively constant (Coetzee, 2013). *Vitis vinifera* berries rapidly synthesize tartaric acid during the early cell expansion and growth phase (Iland & Coombe, 1988) and the early stages of grape berry development (Terrier *et al.,* 2001). They accumulate tartaric acid in the vacuole (Moskowitz & Hrazdina, 1981). Grape berries accumulate tartaric acid in their pulp (Ruffner, 1982), which strongly impacts the taste and organoleptic qualities of the resulting juice and the final product (Ribéreau-Gayon, 1968). Generally, tartaric acid is present in a constant average concentration in the juice of around 5 to 10 g/L (Ruffner, 1982), whereas malic acid juice concentrations fluctuate between 2–6.5 g/L, mostly according to the climatic growing region (Coetzee, 2013).

2.5.3 Malic acid (MA)

Malic acid, of which the chemical structure of the molecule is shown in Figure 2.9, is known to be an active intermediate in grape metabolism and the only high-proportion organic acid actively metabolised during ripening (Sweetman *et al*., 2009). Malic acid accumulates in the first six weeks of berry development by the metabolism of sugars translocated to the grape berry and is broken down to glucose and fructose with a substantial decrease in concentration after véraison (Terrier & Romieu, 2001; Sweetman *et al.*, 2009). After véraison, malic acid is released from the vacuole and

catabolised through different pathways, including the trichloroanisole (TCA) cycle, respiration, gluconeogenesis, amino acid interconversions, ethanol fermentation and included in the production of complex secondary compounds such as anthocyanins and flavonols (Sweetman *et al.*, 2009). It has a significant role in certain anabolic reactions such as dark fixation of carbon dioxide and acid catabolising processes connected to fruit ripening (Ruffner, 1982).

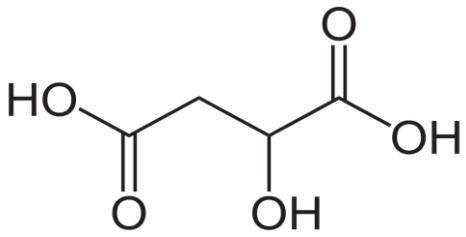

Figure 2.9: The chemical structure of L-malic acid

(www.chemspider.com; accessed 02 March 2018)

Malic acid metabolism is dependent on the physiology of the fruit, the isoforms in which the enzymes are present and, in addition, the subcellular compartmentalisation of the enzymes, and the availability of the substrates (Martínez-Esteso *et al.*, 2011).

2.5.4 Citric acid

Citric acid forms a minor component of grapes and wines, which makes its accurate determination in musts and wines difficult (Peynaud, 1936). Citric acid is an intermediate of the trichloroanisole (TCA) cycle and is widespread in nature (e.g. in lemons) (Chidi *et al.,* 2018). It plays a critical role in the biochemical processes of grape berry cells, bacteria and yeast (Chidi *et al*., 2018). High citric acid levels during fermentation could lead to a slower yeast growth rate (Nielsen & Arneborg, 2007). Therefore, concentrations of citric acid in must and wine prior to malolactic fermentation should be relatively low, between 0.5 and 1 g/L (Kalathenos *et al.,* 1995). Citric acid addition during fermentation influences the acidity and flavour of wines by promoting the perception of “freshness“, while, at the same time, promoting microbial instability and the growth of unwanted microorganisms (Chidi *et al*., 2018). In European countries and Canada, citric acid is most commonly used as an acid supplement during the fermentation process to help winemakers boost the acidity of their wine especially grapes grown in warmer climates and complement a specific flavour or prevent ferric hazes (Bartowsky & Henschke, 2004).

2.5.5 Volatile acidity (acetic acid)

Alcoholic fermentation of grapes usually results in the production of acetic acid (Chidi *et al*., 2018). The primary volatile acid present in wines is acetic or volatile acid, a rather loose term referring to the volatility of acetic acid with steam (Amerine, 1954). Usually, formic, butyric, and possibly other

fatty acids are included as well as acetic acid in wine, but lactic acid is not (Amerine, 1954). Acetic acid is a two-carbon volatile organic acid produced during wine fermentation and is mostly responsible for the sour and vinegary smell and taste in wines (Chidi *et al*., 2018). This process occurs mainly at the beginning of alcoholic fermentation, and again towards the end (Bartowsky *et al*., 2003). Apart from yeast metabolic activity, the involvement of aerobic acetic acid bacteria during fermentation can also produce acetic acid by oxidising ethanol (Ghosh *et al.,* 2012). The critical acetic acid detection threshold in wine is estimated to be approximately 600 mg/L (Chidi *et al*., 2018). However, the normal, desirable acetic acid level in wines is about 100 to 300 mg/L (Ribéreau-Gayon *et al.,* 2006). High volatile acidity in wine presents a major problem, with most wineries recommending the use of lower initial sugar-containing must to reduce acetic acid formation during fermentation (Chidi *et al*., 2018). However, acetic acid concentrations can reach above 1 g/L, depending on environmental factors and the nutritional composition of the must, as well as the influence of spoilage yeasts and bacteria (Bely *et al.,* 2003). Since the aroma threshold for acetic acid varies depending on the wine variety and style, the maximum acceptable limit for most wines is 1.2 g/L (OIV, 2010). However, the volatile acidity of ice wines and botrytised wines can reach a maximum acetic acid concentration of 2.1 g/L (OIV, 2010).

2.5.6 Lactic acid

Lactic acid is an organic acid that also contributes to the overall acidity of wine (Chidi *et al*., 2018). The reason why it is attractive to winemakers is that it is much softer on the palate than malic acid (Robinson & Harding, 2015). Lactic acid concentrations typically average from 1 to 3 g/L in wines (Boulton *et al.,* 2013), but can be higher in wines that have undergone malolactic fermentation, whereby malic acid is decarboxylated to lactic acid (Volschenk *et al*., 2006). Unlike malic and tartaric acid, lactic acid is a softer and milder acid that contributes to a creamier mouthfeel of the wine (Chidi *et al*., 2018).

During winemaking, lactic acid production is usually controlled by sulphur dioxide addition, which suppresses the metabolic activities of lactic acid bacteria such as those belonging to the *Oenococcus* and *Lactobacillus* genera (Osborne *et al.,* 2000). However, small amounts of lactic acid can also be synthesized through cellar practices such as maceration and cold stabilisation (Jackson *et al.,* 1987). While high lactic acid levels present no major problems in wine, lactic acid bacteria are capable of changing the sensorial characteristics of certain wines through the degradation of terpenes and other flavour molecules produced during alcoholic fermentation, as well as producing potentially undesirable aromatic compounds such as diacetyl (Lonvaud-Funel, 1999).

2.6 Factors influencing the synthesis of ascorbic acid

Ascorbic acid biosynthesis in grape berries is influenced by external stimuli such as light, temperature and water (Tamaoki *et al*., 2003; Li *et al*., 2008; Ma *et al*., 2008; Ioannidi *et al*., 2009). Several authors have described climate as having a significant influence on berry composition with temperature determined as the main component of climate affecting the berry composition (Ma *et al*., 2008; Ioannidi *et al*., 2009; Hunter & Bonnardot, 2011). Climate is mainly monitored on three different scales, namely a macroclimatic (regional and mainly long term), mesoclimatic (vineyard area or districts and shorter periods of time) and a microclimatic (climate within or surrounding the plant, mainly monitored in minutes and seconds) (Bonnardot *et al.,* 2004). Ascorbic acid is most sensitive to destruction when the commodity is subjected to adverse handling and storage conditions (Lee & Kader, 2000). Losses are enhanced by extended storage, higher temperatures, low relative humidity, physical damage, and chilling injury (Lee & Kader, 2000).

2.6.1 Light

An important factor influencing the ascorbic acid content in several plant tissues is the light intensity (Gatzek *et al.,* 2002; Tamaoki *et al*., 2003). Light is one of the environmental factors that influence leaf ascorbic acid concentration and is required for photoprotection (Badejo *et al.,* 2009). Although light is not essential for the synthesis of ascorbic acid in plants, the quality and quantity of the light during the growing season have a definite influence on the amount of ascorbic acid formed (Lee & Kader, 2000). Ascorbic acid is synthesized from sugars supplied through photosynthesis in plants. Outside fruit exposed to sunlight contains a higher amount of ascorbic acid than inside and shaded fruit on the same plant (Lee & Kader, 2000). In general, the lower the light intensity throughout the entire growing season, the lower the ascorbic acid content of plant tissues (Harris, 1975). Cruz-Rus *et al.* (2010) reported that grape berries exposed to high (1500 $\mu E.m^{-2}.s^{-1}$) or low (200 $\mu E.m^{-2}.s^{-1}$) light from post-flowering until véraison has different ascorbic concentrations, with ascorbic acid content two times higher in berries exposed to high light in comparison to fruits exposed to low light.

It has to be noted that light and temperature cannot easily be separated as the temperature of grape berries is linearly related to the level of incident radiation, where direct sunlight has a greater heating potential than diffused light (Smart & Sinclair, 1976). Higher temperatures and higher respiration rates usually found in light-exposed berries are, in general, attributed to lower malic acid levels (Coetzee, 2013). DeBolt *et al.* (2006) and Kliewer and Schultz (1964) showed that maximum levels of tartaric acid formation were obtained where berries were fully exposed to the sunlight. The berry size of shaded berries is increased in relation to exposed berries (Coetzee, 2013), thereby decreasing the concentration of the tartaric and malic acid in the berry as a

consequence of dilution (Coetzee, 2013). Higher levels of malic acid in shaded berries can be attributed to lower berry temperatures as well as a decrease in light (Melino *et al.*, 2011).

2.6.2 Temperature

Temperature also influences the composition of plant tissues during growth and development (Coetzee, 2013). Total available heat and the extent of low and high temperatures are the most important factors in determining the growth rate and chemical composition of horticultural crops (Lee & Kader, 2000). Although temperature has been found to have little or no effect on the tartaric acid content of the berries (Kliewer, 1964; Buttrose *et al.,* 1971; Coombe, 1987; Terrier & Romieu, 2001), the malic acid content reveal significant changes according to the seasonal climatic differences (Winkler, 1962; Coombe, 1987; Van Leeuwen *et al*., 2004). Reuther and Nauer (in Lee & Kader, 2000) showed that 'frost satsuma' mandarins contained more ascorbic acid when grown under cool temperatures (20–22°C day, 11–13°C night) than hot temperatures (30–35°C day, 20–25°C night).

The effect of air temperature on the composition of the berry in relation to berry size, soluble solids, malic acid, pH, and titratable acidity, however, vary according to the duration of exposure and stage of berry growth (Crippen & Morrison, 1986). Variations in heat summation during berry development can be related to the seasonal variations in the organic acid and sugar concentrations at harvest (Winkler, 1962; Buttrose *et al.*, 1971). In conditions where there are many days with temperatures above 30°C during flowering and véraison, grapes with a lowered total acidity are produced (Jones & Davis, 2000).

2.6.3 Water

A strong relationship exists between improved grape quality and water deficit before véraison when water deficit probably affects grape quality indirectly (Van Leeuwen *et al.*, 2004). The availability of water to the grapevine, either as rainfall or irrigation, affects the berry composition through grapevine water status (Van Leeuwen *et al.*, 2004; Myburgh & Howell, 2012; Coetzee, 2013). This, in turn, depends on the climate, soil and the training system (Van Leeuwen *et al.*, 2004; Van Leeuwen & Seguin, 2006). The seasonal timing of the water deficit is, however, indicative of the response of the grapevine (Roby & Matthews, 2004; Myburgh & Howell, 2012). The influence of grapevine water status on the organic acid content is mainly indirect, through influencing the physiological responses of the plant (e.g. phenology), the vegetative response (e.g. vigour) and the reproductive responses (e.g. berry size) (Coetzee, 2013). An early water deficit provokes early shoot growth cessation and reduces berry size (Van Leeuwen *et al.*, 2004). Under these conditions, berry sugar and anthocyanin concentrations are increased because of a higher ripening speed, and the total acidity is reduced as berries contain less malic acid (Van Leeuwen *et al.*,

2004). Grape quality is high on the soils that induce water deficit, especially on clayey soils where water deficits occur early in the season but are moderate (Van Leeuwen *et al.*, 2004).

High water availability can also induce vigorous conditions, which firstly influences the berry volume, and secondly the canopy microclimate through shading (temperature and light exposure) (Coetzee, 2013). It has to be noted though that the berry volume does not influence the malic acid or tartaric acid content of the berries, but the total titratable acidity and the pH through dilution (Coetzee, 2013). Shading can contribute to a higher malic acid content due to lower berry temperatures (Dry & Coombe, 2005).

2.6.4 Vineyard management practices

The ultimate objective of canopy management is to obtain a photosynthetic efficient, homogeneous canopy with uniformly and well-distributed shoots of similar vigour, producing healthy, high quality grapes of similar bunch and berry size and with a uniform level of ripeness (Hunter & Archer, 2002). In addition, in order to maintain longevity, growth and development of other parts of the vine must not be impaired (Hunter & Archer, 2002).

Viticultural management practices (i.e. pruning, trellising, canopy management) influence the foliage density and orientation mainly alter the microclimate of the grapevine, which alters the physiology of the vine (Smart *et al.*, 1985). Trellising, leaf removal and pruning strategies all alter the light interception of bunches and, therefore, influence the levels of organic acids found in the berries (Coetzee, 2013). Understanding the growth stages of the plant is beneficial in determining the optimal timing of cultural and chemical practices (Jones & Davis, 2000).

Smart *et al.* (1985) and Terrier and Romieu (2001) reported that higher malic acid concentrations could be found where conditions lead to vigorous vines and high yields. Therefore, altering the light and temperature as a result of dense canopy management, the acids content at harvest may be altered (Smart *et al.,* 1985; Terrier & Romieu, 2001). In the case of tartaric acid, even though it was found by Melino *et al.* (2011) that ascorbic acid accumulation in the berry is light-dependent as higher sun exposure resulted in higher levels, it was also found the conversion of ascorbic acid to tartaric acid is driven by factors that are not responsive to light (Coetzee, 2013). In addition, the accumulation of oxaloacetate was also not responsive to light throughout berry development (Coetzee, 2013).

Studies on the role of climate on grape composition may be challenging to interpret because climate encompasses all the environmental conditions of sunlight, temperature, humidity, and rainfall within a region, all of which play important roles in the growth and development of the vine and berry (Robinson *et al.,* 2014).

2.7 The beneficial antioxidant action of ascorbic acid and sulphur dioxide in winemaking

Sulphur dioxide (SO_2) has important roles in winemaking, in both grape juice and finished wines, including enzyme inhibition and antimicrobial and antioxidant effects (Makhotkina *et al*., 2014). For ascorbic acid to be effective in wine, it must be used in conjunction with SO_2 (Barril *et al.,* 2016). However, limits to sulphite concentrations in wines have been established given health concerns about excessive sulphites and detrimental sensory effects (Monro *et al*., 2012). Complementary antioxidant roles have been ascribed to ascorbic acid, particularly direct oxygen scavenging, allowing the use of combined sulphite or ascorbic acid additions, with a consequent lower sulphite application (Bradshaw *et al.,* 2011). This is not only to ensure that SO_2 is present to allow efficient removal of hydrogen peroxide (H_2O_2) but also to ensure that SO_2 can maintain the microbial stability of wine (Boulton *et al.,* 1999). Complete replacement of SO_2 by ascorbic acid in white wine is not recommended as the aerobic oxidation of ascorbic acid produces hydrogen peroxide, considered to be a significant oxidant in wine (Bradshaw *et al*., 2004). The combination of ascorbic acid and SO_2 has been shown to have a favourable influence on the sensory features of white wines, including aroma, taste and clarity (Kielhöfer & Würdig, 1959; Bauernfeind & Pinkert, 1970). In a Riesling and Chardonnay bottle trial, Skouroumounis *et al*. (2005a) showed that the addition of 90 mg/L of ascorbic acid at bottling had little impact on wine aroma in the first six months. Differences, however, became apparent at three years and five years of bottle age (Skouroumounis *et al*., 2005b). The extent of wine oxidation characters, determined via sensory assessment, was either the same or lower for wines containing ascorbic acid compared with those without added ascorbic acid (Skouroumounis *et al*., 2005b).

Another study utilising Riesling (Table 2.2) showed that ascorbic acid addition (250 mg/L) to wines led to increased perception of fruity aromas and a lower intensity of oxidised aromas (oxidised apple, honey and sherry) when assessed six months after bottling (Morozova *et al.,* 2015). On the palate, the wines with added ascorbic acid were also perceived as less oxidised, less ripe and fresher (Morozova *et al*., 2015). A higher concentration of sulphur dioxide (68 mg/L free SO_2 vs 45 mg/L, Table 2.2) was found to be less effective than a combination of ascorbic acid and SO_2 in preventing negative sensory attributes in wines exposed to higher oxygen concentration during storage (Barril *et al*., 2016).

The impact of bottle closure in the presence of ascorbic acid has been examined in several studies on Semillon, Sauvignon blanc, Riesling and Chardonnay (Table 2.2) utilising a range of ascorbic acid from 40–100 mg/L at bottling (Godden *et al*., 2001; Skouroumounis *et al*., 2005a; Lopes *et al*., 2009). In these studies, the oxygen available to the wine during bottle ageing was from three sources; oxygen dissolved in the wine, oxygen trapped in the bottle headspace (between the

closure and the wine surface) and oxygen that permeated through and/or from the closure (Barril *et al*., 2016).

Table 2.3: Compositional data for white wines utilised for bottle trial studies with ascorbic acid

Wine	Ascorbic acid (mg/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Dissolved oxygen (mg/L)	Headspace oxygen (mg O_2)	References
Semillon	41–44	30	95	0.6–3.2	Not measured Inert gas used during bottling.	Godden *et al*. (2001)
Riesling	0 and 99	29	155	0.8–1.1	Not measured Inert gas used during bottling.	Skouroumounis *et al*. (2005a)
Chardonnay	0 and 92	28	67	1.1–1.5	Not measured Inert gas used during bottling.	Skouroumounis *et al*. (2005a)
Sauvignon Blanc	85	41	132	0.2–2.4	Not measured Inert gas used during bottling.	Lopes *et al*. (2009)
Riesling	0 and 250	45	149	<0.3	0, 2.7, 5.4, 8.2	Morozova *et al*. (2015)

All values measured just after bottling. Each sulphur dioxide concentration was used for wines with and without ascorbic acid, and four different headspace volumes were used for each ascorbic acid and sulphur dioxide treatment. Only screw cap closures were utilised in this study (adapted from Barril *et al*., 2016).

2.8 Pinking potential of white wines and ascorbic acid use

Some white wines undergo a subtle colour change called pinking after processing that makes the wine appear slightly or noticeably pink (Lamuela-Raventó *et al.,* 2001). This problem, considered serious by many winemakers, appears after a wine has been stored and then exposed to small amounts of air or oxygen (Lisjak, 2007). Pinking is a term that describes the appearance of a salmon-red blush in white bottled wines produced exclusively from white grape varieties (Andrea-Silva *et al*., 2014). Although, with seasonal and regional variations, pinking has been observed worldwide, with predominance in white wines produced from the *Vitis vinifera* L. grape varieties such as Chardonnay, Chenin blanc, Crouchen, Muscat Gordo Blanco, Palomino, Riesling, Sauvignon blanc, Semillon, Sultana, and Thompson seedless (Simpson, 1977).

Pinking is mainly observed when white wines are produced under reducing conditions (Simpson, 1977). The pinking phenomenon is usually observed after bottling and storage of white wines or

after alcoholic fermentation, although sometimes it occurs as soon as the grape must is extracted (Simpson *et al.,* 1982). Susceptibility to pinking of reductively produced wines may be effectively overcome by maintaining adequate levels of free sulphur dioxide, especially when ascorbic acid is used (Clark *et al.,* 2008). It has been proposed that white wine pinking can be a result of the rapid conversion of accumulated flavones to the corresponding red flavylium salts formed from the hydrolysis of leucoanthocyanins (Zoecklein *et al.,* 1990). There are various preventive or curative oenological treatments for the pinking problem, including adding polyvinylpolypyrrolidone (PVPP) associated with gelatine or increasing the redox potential using ascorbic acid in the pre-bottling stage (Lamuela-Raventó *et al*., 2001).

Pinking is thought to be due to oxidative changes in white wines when exposed to oxygen (Singleton *et al.,* 1979), although the compounds responsible for the pinking phenomenon are unknown (Andrea-Silva *et al*., 2014). The compounds responsible for pinking has not been identified, but it is thought to be a phenolic chromophore (Margalit, 2004). According to Jones (1989), the pinking phenomenon can be caused by at least ten different compounds plus polymeric material, although several of these possibilities appear to be unfeasible (Lisjak, 2007). There are, however, strong indications that the pink compounds could be a derived from grape reaction products (GRP), the reaction product of glutathione with caftaric acid-*o*-quinone, which is generated by enzymatic oxidation of caffeoyl- and *p*- coumaroyl tartaric acids (Singleton *et al.,* 1985).

2.9 Conclusion

From the above, it is clear that there is a gap in the factors that contribute to ascorbic acid derivatives in grapes at harvest. In order to further understanding and knowledge of these factors, different approaches may be considered. Several factors influence the acid content in the grape berry. These include climatic factors such as temperature, which is the main climatic factor contributing to the differences in the content of these acids. Although the climate mainly drives the temperature, canopy manipulation can alter not only the microclimate of the grapevine with subsequent changes but also the light interception of the grape bunches. Therefore the aim of the study was to monitor ascorbic acid derivative levels in Sauvignon blanc (*Vitis vinifera* L.) during berry development and in wine under South African weather growing conditions.

CHAPTER 3:
MATERIALS AND METHODS

3.1 Location of the experimental sites

The work was done during the season of September 2018 to March 2019 on rooted grapevines *Vitis vinifera* L. cv. Sauvignon blanc. Two study sites were selected in two diverse climatic regions – Wellington (33.5778°S, 19.0202°E) and Elgin (34.2169°S, 19.0560°E). Sauvignon blanc vines in Wellington were planted in 1983 and those in Elgin in 2003, with 2.5m x 1.5m vineyard spacing. The vines were spur pruned to two buds per spur on a four-strand double lengthened Perold system (SDLP). Grapevines were sampled on one farm in the Wellington district, Schalk Burger & Sons, and one farm in the Elgin district, Paul Wallace Farm. Canopy management practices were carefully planned and performed at E-L 29 to create a favourable microclimate towards berry composition. The timing of canopy management was adapted in such a way that the physiological cycle of the grapevine was favourable so that the required grape composition and wine style could be achieved.

3.1.1 Wellington

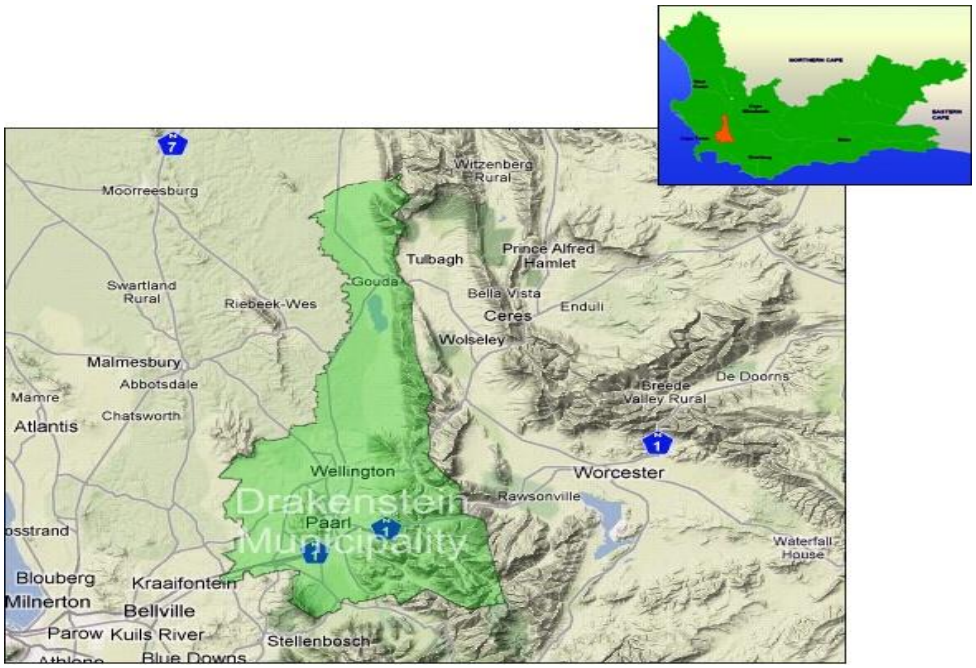

Figure 3.1: Wellington in Drakenstein municipality in Western Cape

Source: Western Cape Province

Wellington (Figure 3.1), is a town in the Western Cape Winelands, a 45-minute drive from Cape Town, in South Africa. The town is located 75 km north-east of Cape Town, reached by the N1

highway and the R44. Wellington officially falls under the Drakenstein local municipality. It usually receives about 585 mm of rain per year, with the lowest rainfall (10 mm) in February and the highest (105 mm) in June (South Africa Explorer, 2017). Wellington has a Mediterranean climate with February being the warmest month with an average maximum day temperature of 30°C and an average minimum night temperature of 17.7°C. July is the coldest month, with an average maximum day temperature of 14.8°C and an average minimum night temperature of 6.3°C (South Africa Explorer, 2017).

3.1.2 Elgin

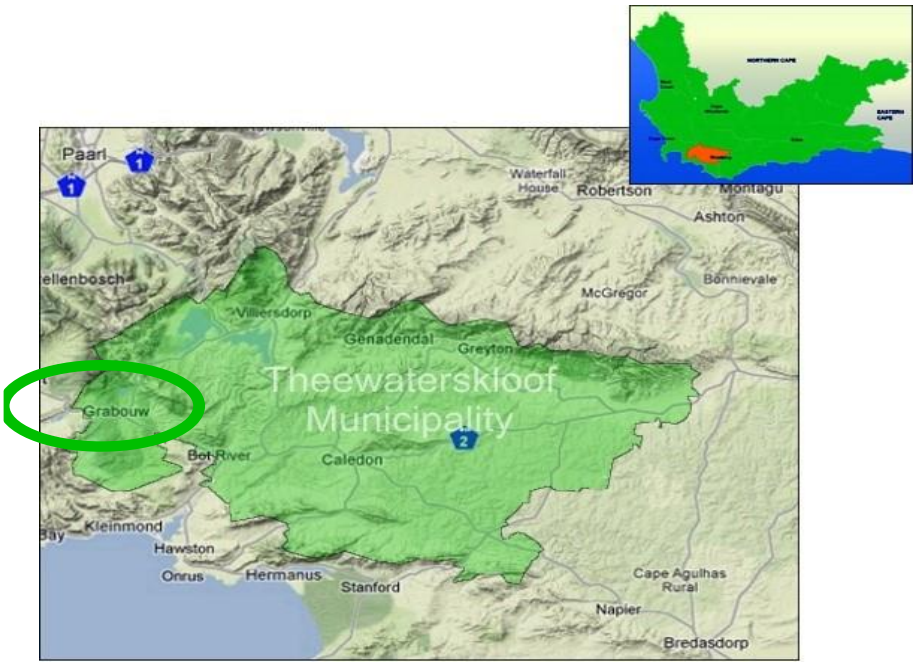

Figure 3.2: Grabouw in Theewaterskloof municipality in Western Cape

(Source: Western Cape Province)

Elgin (Figure 3.2), is situated close to the Southern Cape Coast between Sir Lowry's Pass in the south-east (outside Somerset West) to Bot River (en route to Hermanus) and Villiersdorp to the north-west in the Overberg region. It is about 70 km east of Cape Town, just beyond the Hottentots-Holland mountain range and is centred around the village of Grabouw. The Elgin Valley is separated from the sea by a narrow range of mountains with altitudes ranging from 300 metres to 600 metres above sea level. These conditions create a cool climate, which is essential for the production of deciduous fruits (Stofberg, 2018). Grabouw also has a Mediterranean climate with an annual rainfall of about 990 mm. The highest rainfall is usually during June (long-term mean of 168 mm) and the lowest rainfall in February (22 mm). February is also the warmest month with an average maximum day temperature of 24.8°C and an average minimum night temperature of 14.1°C. July is the coldest month, with an average maximum day temperature of 14.8°C and an

average minimum night temperature of 6.4°C (South Africa Explorer, 2017). The area is well-known for its agriculture and tourism, and many towns along the coast and in the interior are popular destinations for tourists and form part of an important agricultural hub in the Western Cape Province (Tswane *et al.*, 2014).

3.2 Treatments

3.2.1 Viticultural treatments

Four vineyard blocks of Sauvignon blanc were selected (two on each farm - described above) with different row orientations (North-South, East-West). The sizes of the vineyard blocks were as follows: Wellington north–south block was 2.3 hectares and the east–west block was 1.2 hectares. In Elgin, the north–south block was 1 hectare, and the east–west block was 1.8 hectares. Wellington's north-south block had 115 grapevines per row, and the east–west block had 80 grapevines per row. Elgin's north–south block had 78 grapevines per row, and the east–west block had 106 grapevines per row. On each farm, six rows in each vineyard block were selected randomly (North-South and East-West row orientation) for the experiment and marked using a danger tape. Three of the six rows were selected as treatments for canopy management practices such as suckering at 10 cm shoot lengths (the removal of unwanted shoots from cordons or arms or from nodes on spurs or canes) on both farms, (Wellington was suckered at the end of September and Elgin was suckered at the end of October). Suckering and leaf removal (three to four leaves removed at the bunch zone depending on the canopy density at E-L 34 phenological ripening stage) were done at the sides that received morning sunlight on both farms and in all the four vineyards blocks so that vine bunches were not exposed to too much afternoon sunlight. All the suckered rows were also topped (when shoots were higher than the anchor pole), leaves removed at the bunch zone (E-L 34) and the vine stem cleaned (at all the stages the ripening phenological ripening stages mentioned).

The other three rows of the six rows in each vineyard block (north–south and east–west row orientation) on each farm were left unhindered (control), and no canopy management practices were performed. The treatments were as follows:

Table 3.1: Treatment delineation for Wellington and Elgin

Treatment 1: North-South row direction; Wellington and Elgin		Treatment 2: East-West row direction; Wellington and Elgin	
Row 1 (Wellington: 115 vines/row; Elgin 78 vines/row)	Non-suckered	Row 1 (Wellington: 80 vines/row; Elgin 106 vines/row)	Non-suckered
Row 2 (Wellington: 115 vines/row; Elgin 78 vines/row)	Suckered	Row 2 (Wellington: 80 vines/row; Elgin 106 vines/row)	Suckered
Row 3 (Wellington: 115 vines/row; Elgin 78 vines/row)	Non-suckered	Row 3 (Wellington: 80 vines/row; Elgin 106 vines/row)	Non-suckered
Row 4 (Wellington: 115 vines/row; Elgin 78 vines/row)	Suckered	Row 4 (Wellington: 80 vines/row; Elgin 106 vines/row)	Suckered
Row 5 (Wellington: 115 vines/row; Elgin 78 vines/row)	Non-suckered	Row 5 (Wellington: 80 vines/row; Elgin 106 vines/row)	Non-suckered
Row 6 (Wellington: 115 vines/row; Elgin 78 vines/row)	Suckered	Row 6 (Wellington: 80 vines/row; Elgin 106 vines/row)	Suckered

Wellington N–S row direction 115 vines per row; Elgin N–S row direction 78 vines per row

3.3 Sample handling up to harvest

Sampling and transportation were performed early in the morning (04:00–10:00) during the cooler part of the day. Each of the rows, suckered or non-suckered, were sampled separately with 900 berries per row, (hence 900 x 6 = 5 400 berries for north–south and east–west suckered and non-suckered vineyard rows), sampled from bunches randomly (top, middle, bottom and back of the bunch) from each row across the six rows per vineyard block (north–south or east–west row orientation) berries pulled off from the septum when sampled. The 900 sampled berries from each row, suckered or non-suckered rows on each of the farms and on each row, were divided by three to get three replicates per row (Figure 3.4). Berries were sampled at five different phenological stages (Figure 3.3), E-L 31 (berry pea size), E-L 33 (berries still hard and green), E-L 35 (véraison), E-L 37 (berries not quite ripe) and E-L 38 (berries harvest ripe) (Figure 3.3). The date of the beginning of each phenological stage was determined visually for every block. The berries were placed into plastic bags for transportation to minimise damage and kept in a cooler box on ice to minimise temperature fluctuations.

Figure 3.3: Berry sampling stages.

The numbers 31 to 37 represent the corresponding grape berry development periods of E-L system of Coombe (1995)

3.4 Sample preparation

Fresh samples were prepared and analysed on the day of sampling on each of the farms. After sampling, 300 berries (from the 900 berries per vineyard row), were put into a 5 L jug and crushed to obtain the juice. In preparing the juice, dry ice was used to cover the extracted juice in order to isolate the juice from oxygen. The juice was immediately transferred to 15 mL falcon tubes and filled to capacity. The tubes were pre-marked suckered or non-suckered (1.1-1.3 to 6.1-6.3). The filled falcon tubes were stored in a cooler box with ice. The remaining juice was used to do standard analysis at the Western Cape Department of Agriculture, Elsenburg Wine Cellar for pH, TA using Lasec (Benchtop pH meter, South Africa) and balling (wine machinery balling meter). The rest of the samples were taken to the Central Analytical Facilities (CAF) laboratories at Stellenbosch University for analysis of ascorbic acid, dehydroascorbic acid, 2,3-diketogulonic acid (DKG) and L-threonate, using ultra-performance liquid chromatography (UPLC) coupled to a Xevo TQ-MS mass spectrometer.

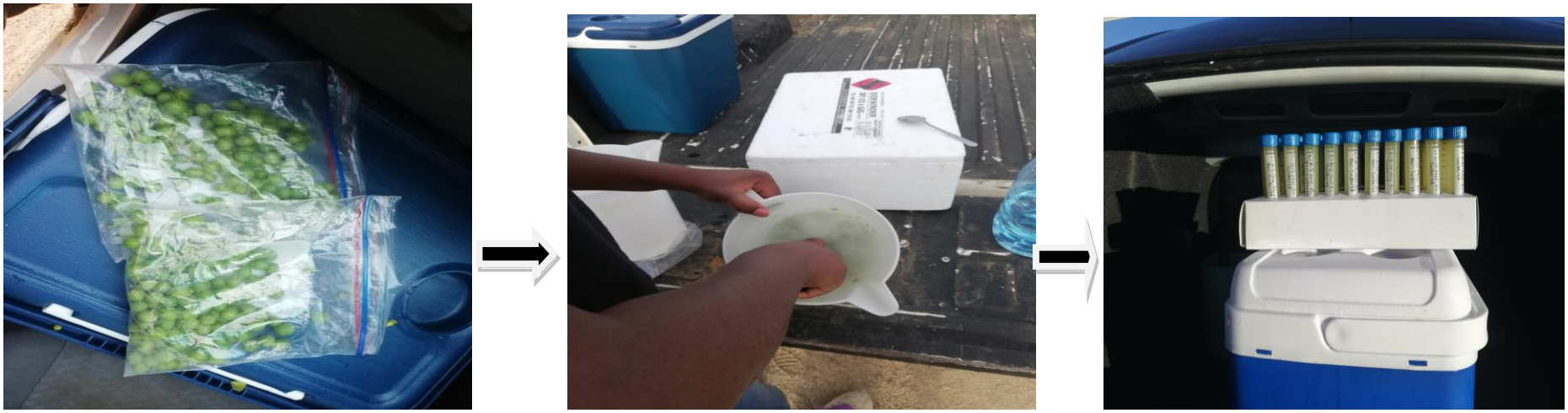

Figure 3.4: Sample preparation procedure

3.5 White wine preparation

Wellington grapes for the season 2018-2019 were harvested at the end of January and the Elgin grapes for the same seasons were harvested in the middle of March. Grapes were hand-harvested by farmworkers in the morning (07:00–12:00) on the day of harvest, and the crates from the rows were marked. The treatments that were suckered were mixed, and the non-suckered treatments were also mixed for both the north–south block and the east–west treatments on each farm. Upon the arrival of the grapes at the cellar, a delivery note was completed for all the treatments. Labels with cellar numbers were supplied for each treatment to keep track of the treatments, then moved to cold storage (5°C temperature) and processed the next day.

Figure 3.5: Grapes received at the cellar in 20 kg crates

3.6 Winemaking style

Wines were made at the ARC Nietvoorbij cellar according to the standard Nietvooribij white winemaking protocol. Grapes were crushed, the juice and skins immediately pressed at up to 1 Bar. Each pressing was completed before the next treatment was crushed. No skin contact was applied to the treatments. To improve sedimentation, 0.5 g/hL *Ultrazym* (Novozymes) and 50 mg/L SO_2 (Table 3.3) was added to the Sauvignon blanc juice. A sample of juice was taken for WineScan analysis (pH, titratable acidity, sugar/balling and SO_2) and the SO_2 level was adjusted to a total of 50 mg/L, if necessary. Thereafter, the juice was allowed to settle at 14˚C. The clear settled juice was racked off the lees by siphoning into a fermentation container, and the volumes of the juice and wine lees noted and recorded in the container card and the cellar book.

The juice was inoculated at 14˚C with a rehydrated pure yeast (VIN 13) at a dosage of 30 g/hL, as well as the addition of 50 g/hL of diammonium phosphate (DAP). After inoculation, the juice continued to ferment at 14˚C, and on the third day of fermentation,

75 g/hL (i.e. 10 mL/L of 7.5% bentonite solution) of bentonite was added to the fermenting juice for protein stabilisation. Close to the end of fermentation, samples were taken under CO_2 gas and analysed for the sugar content. After fermentation was completed, wines were racked off the yeast lees. The free (FSO_2) and total (TSO_2) SO_2 were tested and adjusted to 45 mg/L FSO_2 (in accordance with the analysis), and then the wines were cold stabilised at 0˚C for at least two weeks. After cold stabilisation, wines were filtered using filter mats (K900 and EK) and bottled in green bottles (see the section on bottling) into nitrogen-filled wine bottles at room temperature.

Table 3.2: Conversion table

Product name	Dosage	Conversion	Dilution
Yeast (VIN 13)	30 g/hL	0.3 g per L or kg	
Enzyme (*Ultrazym*)	0.5 g/hL	1 mL per L	1%
Sulphur dioxide (SO_2)	50 mg/L	1 mL per L	5%
Nutrition; Diammonium Phosphate (DAP)	50 g/hL	1 mL per L	50%
Protein stabiliser (Bentonite)	75 g/hL	10 mL per L	7.5%

Dosage: the addition of an ingredient in a measured dose; conversion: conversion between different units of measurement for the same quantity; dilution: decreasing the concentration of a solute in a solution

3.7 Bottling the wine

Before bottling the wine, all bottles were washed with iodine and placed upside-down the day before bottling on a drying rack. Bottle caps were soaked in a diluted SO_2-water solution and shaken off well before use. Filter mats (K900 and EK) were pre-packed and washed with SO_2 and citric acid. A filter aid (*Kieselguhr*) was added to the water used to rinse the filters, and some of it was also added to the wine canister to aid filtration. The first bottle of each treatment that is filled with wine was discarded to prevent cross-contamination between treatments. Simultaneously, the following bottle was filled with nitrogen gas (N_2) to replace the oxygen in that bottle while filling it with the wine. After a bottle was filled to the top, a cap or roll-on seal was placed on the bottle by using a roll-on machine. After bottling was done, the bottles were coded according to their treatments and the wines stored at 14˚C until the annual wine sensory evaluation in September 2019.

3.8 Test for pinking potential of Sauvignon blanc wine

The Sauvignon blanc wines for the 2018–2019 vintage from Wellington and Elgin were also tested for their potential to pink at the Cape Peninsula University of Technology, Agricultural Hub, using a UV-1600 PC spectrophotometer after bottling. The pinking phenomenon is usually observed after bottling and storage of white wines or after alcoholic fermentation,

although sometimes it occurs as soon as the grape must is extracted (Andrea-Silva *et al*., 2014). The visible absorption spectrophotometer of a Sauvignon blanc, induced by standing in a glass for 24 h to determine the pinking potential of the wines, showed that the colour of the wine changed from a clear pale yellow to a pale salmon colour with a clear increase in the absorbance in the visible region. The pinking susceptibility is a quantitative measure of the degree of pinking following the addition of a small amount of hydrogen peroxide (H_2O_2) (1000 µL of 0.072% H_2O_2). When the Sauvignon blanc wines with the potential to pink are exposed to oxygen, they turn pink.

One hundred and twenty microliters of 30% hydrogen peroxide (H_2O_2) was added to a 50 mL volumetric flask and filled up to the mark with distilled water to obtain a 0.072% hydrogen peroxide solution. One mL of 0.072% H_2O_2 was added to each sample. The falcon tubes were closed immediately after the addition of H_2O_2 to prevent any oxidation and mixed very gently by tilting up and down. The samples were allowed to stand for eight hours in a dark cupboard. The optical density at 500 nm of samples was read the next day, measured against a control sample with no added H_2O_2 used to zero the spectrophotometer at 500 *nm* (Figure 3.5) (SAWLA, 2012).

Figure 3.6: Spectrophotometric determination of pinking potential in wine

3.9 Analyses protocols

3.9.1 Liquid chromatography-mass spectrometry (LC-MS) analysis

A Waters acquity ultra-performance liquid chromatography (UPLC) coupled to a Xevo TQ-MS mass spectrometer (MS/MS) was used for high-resolution UPLC-MS/MS analysis. Separation was achieved on an Acquity HSS T3 column (2.1 x 100 mm; 1.7 µm particle size) at 40˚C and a flow rate of 0.25 mL/min. Data was acquired with multiple reaction monitoring (MRM) using electrospray negative ionization. The operating parameters used were as follows: capillary voltage, 3.5 V; cone voltage range, 10-35 V; collision energy range, 5-40 eV; source temperature, 140˚C; desolvation temperature, 400˚C; desolvation gas, 800 L/h

and cone gas, 50 L/h. An injection volume of 5 µL was used, and the mobile phase consisted of water acidified with 0.1% formic acid and acetonitrile acidified with 0.1% formic acid.

3.9.2 Statistical analysis

Statistical differences between treatments were determined by applying standard principal component analysis (PCA) and analysis of variance (ANOVA) methods (Appendix A). All values were expressed as means ± S.D. A PCA was used to look for the relationships, groupings and patterns in the data. The observations in the PCA are the mean values of the treatment combination of the main treatment and the row orientations observed in five stages at each area. PCA was performed using XLSTAT software (version 7.5.2, Addinsoft, New York, USA).

A split-plot experimental design with treatments as main plot factor and stages as sub-plot factor was used in the analysis. Combined ANOVA of farms and row orientations was performed. Homogeneity of variance was verified by Levene's test (Levene, 1960) and normality of standardized residuals verified by the Shapiro Wilk test (Shapiro & Wilk, 1965). ANOVA was performed using the GLM procedure of SAS statistical software (Version 9.4, SAS Institute Inc., Cary, NC, USA). Fisher's least significant difference was calculated at the 5% level of significance to compare treatment means (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

CHAPTER 4:
RESULTS AND DISCUSSION

4.1 Ascorbic acid derivatives in Wellington and Elgin

The differences in behaviour/trends of the ascorbic acid derivatives between the two regions, Wellington and Elgin, were measured. The figures show the responses in DHA, DKG and L-threonate concentration levels during berry development to different treatments: suckered versus non-suckered, and north to south versus east to west row orientations. The results show the levels of ascorbic acid derivatives in Sauvignon blanc during berry development until harvest, determination of trends in the development of ascorbic acid derivatives between Wellington and Elgin, principal component analysis (PCA) of Sauvignon blanc wines for Wellington and Elgin, determination of the pinking phenomenon associated with Sauvignon blanc cultivar between two climates and other ripening grape parameters such as pH, titratable acidity TA and balling.

4.2 The levels of ascorbic acid derivatives in Sauvignon blanc (*Vitis vinifera* L.) during berry development until harvest

Figure 4.1 depicts the interaction between row direction and stage of ripeness for the ascorbic acid derivatives on each farm during the ripening seasons. Figure 4.1 (A & B) (Appendix A), shows the interaction for the Wellington (A) and Elgin (B) in the north–south and east–west vineyard blocks. The results for Wellington regarding row orientation and phenological stage interaction (Figure 4.1 (A)), shows that DKG and L-threonate concentrations were higher at E-L 32 for both the north–south and east–west row orientations. The ascorbic acid derivatives varied quantitatively from E-L 32 to E-L 38 on both farms. At the conditions with a lower DHA and DKG, the L-threonate concentration derivative was high. Generally, DKG and L-threonate concentrations significantly differed ($p \leq 0.05$) between E-L 32 to E-L 38, which is the result of DHA instability in aqueous solutions. This was in line with what was observed by Deutsch (2000) that DHA is an unstable molecule and according to most literature reports it is lost upon hydrolysis of the lactone ring of DHA, resulting in the formation of DKG. At physiological pH, DHA is rapidly hydrolysed to DKG (Deutsch, 1998a). Under conditions of oxidative stress and after being hydrolysed to DKG, DHA rapidly degrades to 5- and 4-carbon species (Deutsch, 1998b). Data in this study clearly show that there were different concentration of derivatives between the blocks on each farm and between the farms. Ascorbic acid readily reacts with reactive oxygen species, resulting in the formation of monodehydroascorbate. Monodehydroascorbate is unstable and, if not rapidly reduced, is disproportionate to DHA (Gallie, 2013). L-threonate, on the other hand DKG, is quite stable in an oxidative environment (Penney & Zilva, 1945), and it was

reflected by the higher derivative concentrations at all ripeness stages on each farm, for both row orientations (north–south and east–west).

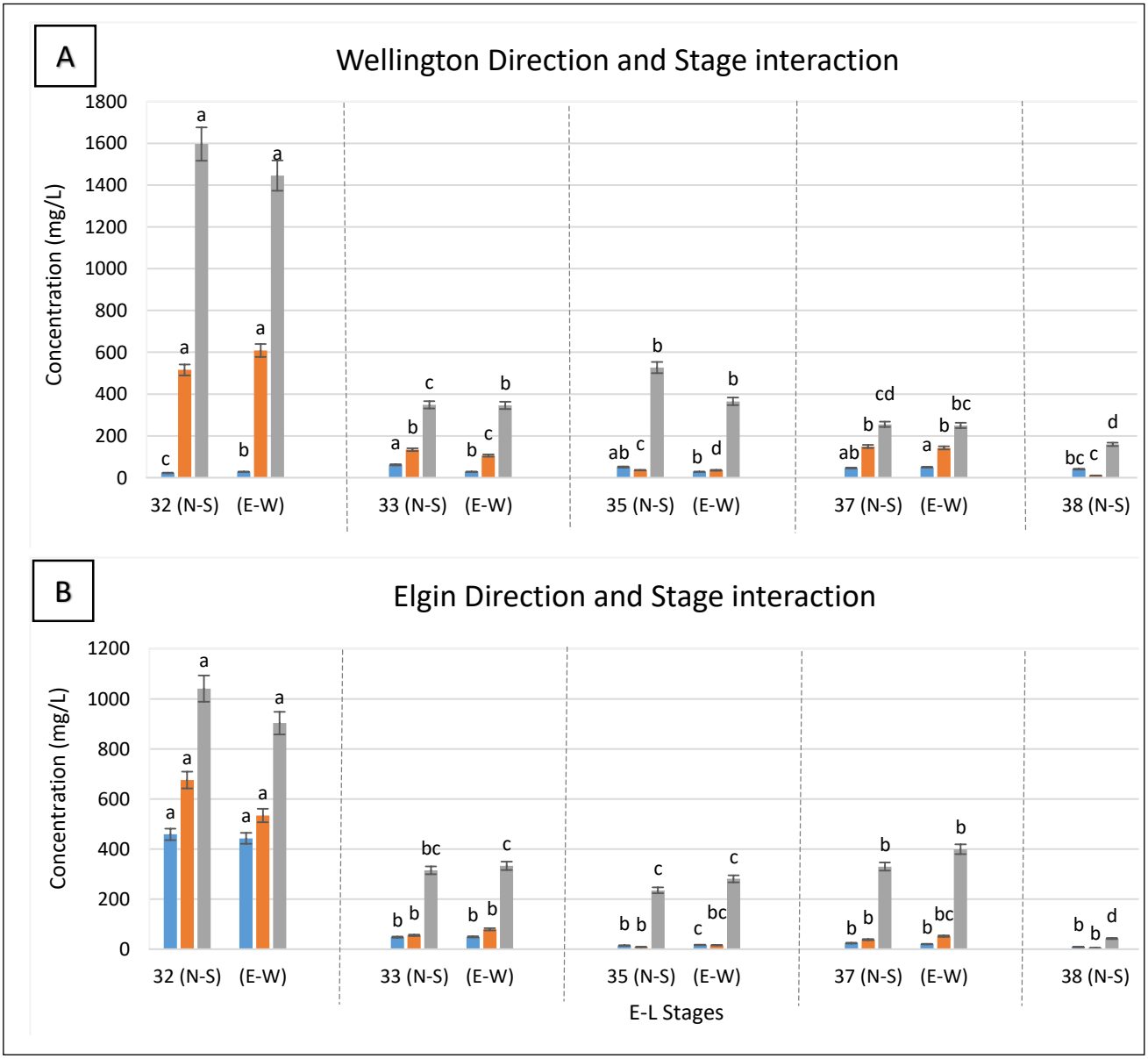

Figure 4.1: Effect of row orientation and stage of ripeness (D x S) on ascorbic acid derivative during five ripening stages E-L 32 to 38 at Wellington (A) and Elgin (B) north–south and east–west rows.

Data are given in means, and values with different letters between different ripeness levels and row directions for each component are significantly different ($p \leq 0.05$)

The results for Elgin (B) orientation and phenological stage interaction, were similar to those obtained from Wellington, with E-L 32 having the highest concentration of derivatives for both north–south and east–west row orientations. Ascorbic acid derivates varied throughout ripening. One reason for the variability in the yield of these derivatives could be their possible degradation due to the susceptibility of ascorbic acid to oxygen. Interestingly both Wellington and Elgin at E-L 32 had the higher concentrations of ascorbic acid derivatives, which could be because the grape berries are still at their greenest stage and the highest concentration of other acids protects against the degradation of derivatives.

Ascorbic acid and its derivatives accumulation patterns during fruit development vary among different plant species (Cruz-Rus *et al.,* 2012). These factors likely contribute to differences in the concentration of ascorbic acid derivatives reported in the literature and this study. According to Gökmen *et al*. (2001), the biologically active forms, ascorbic acid and dehydroascorbic acid have equal antiscorbutic activity. Although ascorbic acid is the more highly reduced species in the ASC-DHA pair, DHA appears to have antioxidant properties of its own, beyond that of ascorbic acid. It has been shown, for instance, that DHA is better than ascorbic acid at protecting low-density lipoprotein from oxidation by cupric ion (Retsky, Freeman & Frei, 1993). Kiwifruit, apple, and peach synthesize ascorbic acid primarily during early fruit development (Bulley *et al*., 2009; Imai *et al*., 2009; Li, Ma, Liang, Li & Wang, 2010). In the tropical fruit, acerola, which contains high amounts of ascorbic acid, the ascorbic acid content decreases rapidly with fruit ripening (Badejo *et al*., 2009). Some fruits such as strawberries, grapes and tomatoes continue to accumulate ascorbic acid during late fruit development (Agius *et al*., 2003; Ioannidi *et al*., 2009; Cruz-Rus *et al*., 2010; Badejo *et al*., 2011). Chemical and biochemical degradation of ascorbic acid occurs through the pathway from ascorbic acid to DHA to diketogulonic acid (DKG), the first reaction being reversible and the second irreversible (Margolis, Paule & Ziegler, 1990).

The DHA initially yields DKG, which is then oxidised to L-threonate, possibly being the reason that DHA was detected at lower concentrations while the DKG and L-threonate were present at higher concentrations. This evidence is in agreement with that of Shimada and Ko (2008) showing that ascorbic acid oxidase catalyses the oxidation of ascorbic acid to yield dehydroascorbic acid, followed by the decomposition of DHA to DKG and other derivatives. The immediate oxidation product of ascorbate is DHA, which may be quickly and irreversibly hydrolysed to DKG (Dewhirst & Fry, 2015) as a result of enzymatic or non-enzymatic reactions, or is reduced to ascorbic acid by dehydroascorbate reductase, which uses glutathione (GSH) as a reductant (Cruz-Rus *et al*., 2011; Ren *et al*., 2013). Saito and Kasai (1969) administered l-[1-^{14}C] ascorbic acid to immature grape berries and discovered that after 24h of metabolism, 72% of the ^{14}C in the soluble fraction was recovered as tartaric acid with most of the ^{14}C in the carboxyl carbon. Another study by Saito and Loewus (1979), showed that tartaric acid labelling from ascorbic acid in grapes was highest in days preceding or at anthesis but declined with berry maturity. Additionally, a part of oxidized ascorbic acid can be further catabolised to smaller molecules such as l-tartaric acid, l-L-threonate, l-glyceric acid, and oxalic acid (Cruz-Rus *et al*., 2011 Ishikawa *et al.,* 2006). According to Huang and Deng (2014), oxalic acid, l-L-threonate, and l-tartaric acid are the main products of ascorbic acid catabolism. DHA can be hydrolysed to DKG or oxidised to a range of products such as l-L-threonate, oxalic acid (OxA), and their esters (Truffault *et al*., 2017). If the exclusion of ROS limits oxidation, DHA in aqueous solution predominantly

undergoes hydrolysis to form DKG. This hydrolysis is probably irreversible *in vivo* (Wechtersbach *et al.,* 2011). DKG itself can be oxidised into an unknown compound ('H'), which itself can be further oxidised to L-threonate; therefore, DKG can act as an antioxidant like ascorbic acid and DHA (Parsons & Fry, 2012).

The concentration of ascorbic acid derivatives in this study declined as fruit developed. Okuse (1981) reported that a high concentration of ascorbic acid from E-L 31, reaching a plateau during fruit maturation (E-L 37 to E-L 38). However, in the study changes in ascorbic acid derivative concentrations during consecutive grape ripening stages were not always significant. Gest *et al.* (2012) reported that the concentration of ascorbic acid derivatives of a plant cultivar varies between different tissues of the same plant, and between cultivars of the same species.

Bradshaw *et al*. (2011) reported that studies conducted on the fate of the DKG either investigate the direct degradation of DKG or its degradation after formation from DHA. DKG generates a wide variety of compounds via numerous mechanisms, including decarboxylation, oxidative decarboxylation, dehydration, keto-enol rearrangements, cyclization, and benzylic rearrangements (Bradshaw *et al*., 2011). L-threonate, as the end product of ascorbic acid catabolism, indicates high ascorbic acid metabolism at all five ripening stages in this study for both farms (Figure 4.1, A & B). L-threonate, on the other hand, is quite stable in an oxidative environment (Deutsch, 1998c). Oxalic acid and L-threonate are more stable, probably explaining why they are better-known end-products of ascorbate degradation (Yang & Loewus, 1975). In summary, DHA, DKG and L-threonate concentrations for the Wellington and Elgin districts varied with ripeness (E-L 32 to E-L 38) for both row orientations and stages of ripeness.

4.3 Determination of trends in the development of ascorbic acid derivatives between Wellington and Elgin

The differences in behaviour/trends of the ascorbic acid derivatives between the two regions Wellington and Elgin, were measured (Figures 4.3–4.5) (Appendix B & C). The figures below show the responses of DHA, DKG and L-threonate concentration levels during berry development to different treatments (suckered versus non-suckered, north–south versus east–west row orientations between two climatic regions) that were done. DHA concentrations for Wellington grapes remained at relatively constant concentrations throughout all the stages of ripeness (E-L 32 to E-L 38) for north–south orientated suckered blocks (Figure 4.2). The east–west suckered blocks, however, had significantly higher concentrations of DHA at E-L 32. The results for Elgin showed high levels of DHA at E-L 32, whereafter it decreased from E-L 33 to E-L 38.

The DKG derivative was also observed during the ripening period between E-L 32 to E-L38 (Figure 4.3). The results showed that DKG was the second derivative to have a higher concentration of ascorbic acid catabolism after DHA, which was the lowest. Both Wellington and Elgin (Figure 4.3) had higher concentrations of DKG at all the E-L stages. This result for DKG as the second derivative of ascorbic acid catabolism after DHA could be the result of having higher concentrations of it as the second derivative. The treatments of suckered and non-suckered vines with north–south and east–west, did not have an impact on the DKG derivative concentrations.

The L-threonate, as one of the final products of ascorbic acid catabolism, was also observed from E-L 32 to E-L 38 (Figure 4.4). L-threonate had the highest concentration of ascorbic acid derivatives at all the E-L stages. As the byproduct of DKG, both farms had the highest concentrations of threonic acid derivative at all the ripening stages, Wellington having the highest concentrations at all the stages than Elgin at E-L 32 (Figure 4.4). The L-threonate ascorbic acid derivative concentrations were very low at E-L 38 for both farms, which could be the results of the increase of sugar content and a decrease in acid during the ripening period of grapes.

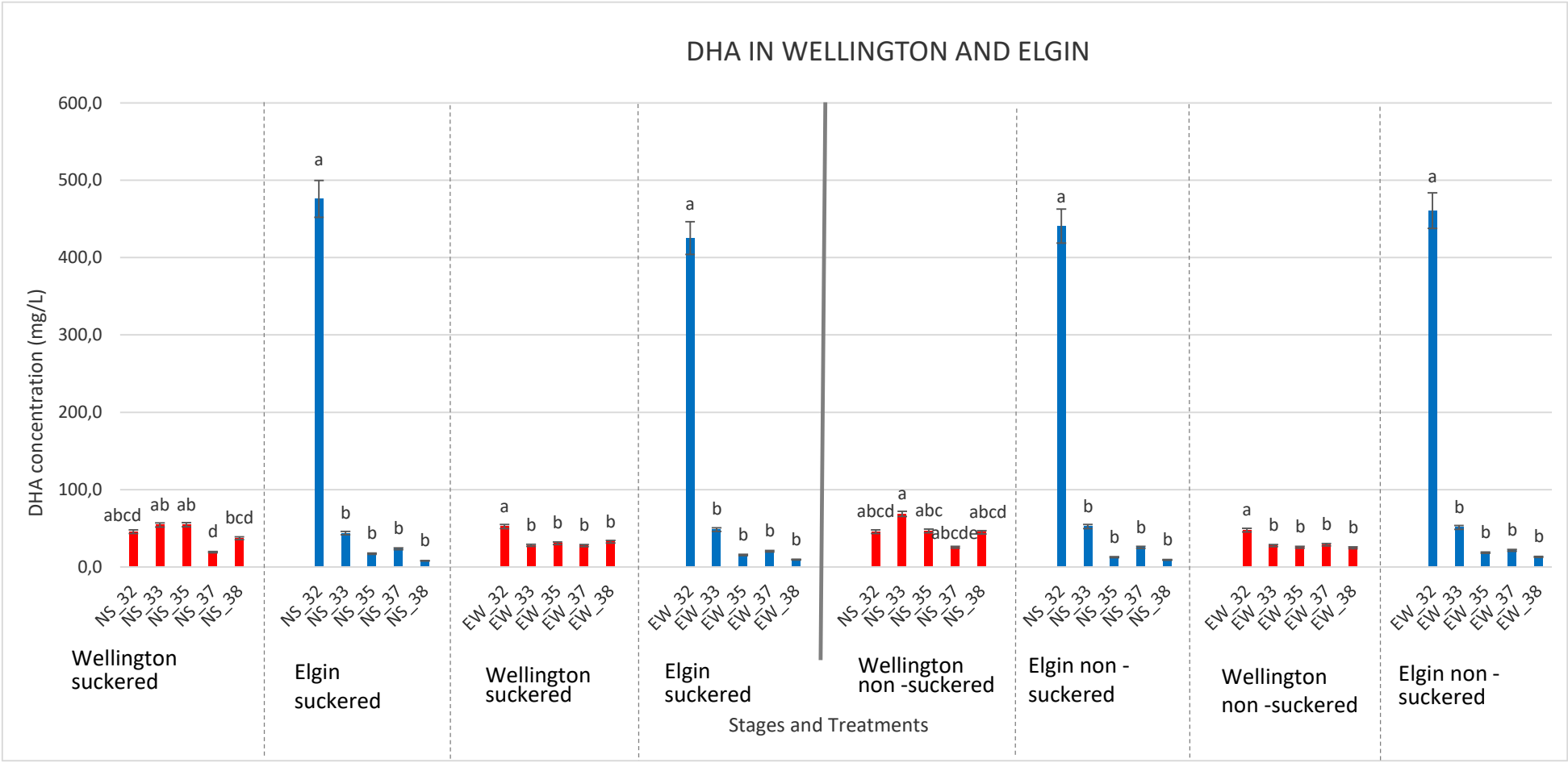

Figure 4.2: Dehydroascorbic acid (DHA) derivative accumulation between two climatic regions Wellington (Red) and Elgin (Blue) for the treatments suckered and non-suckered, and north–south and east–west row orientations

The results show the data collected per region and individual treatments on each farm. Data are given in means and bars with same letters show p-value is significant at $p \leq 0.05$ and letters that are not the same show insignificant when $p \geq 0.05$

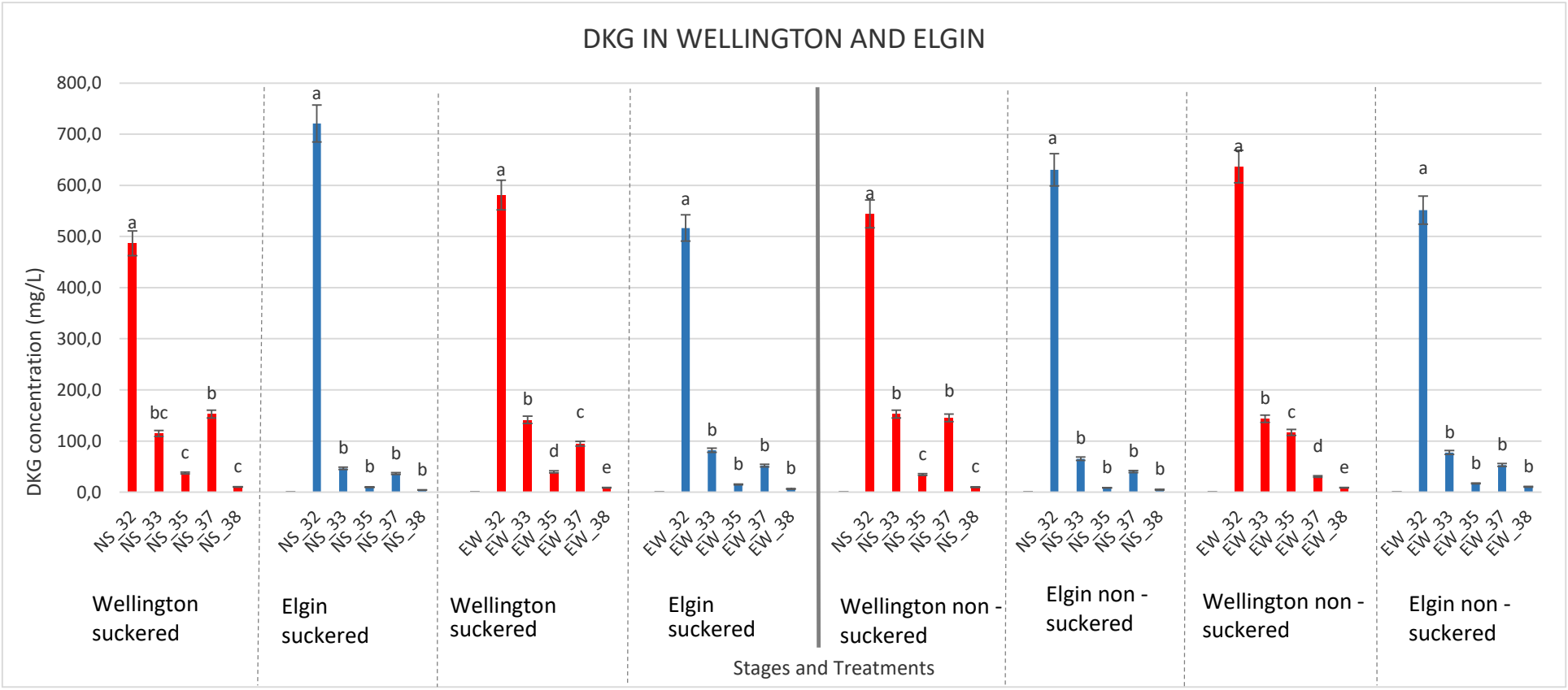

Figure 4.3: Diketogulonic acid (DKG) derivative accumulation between two climatic regions Wellington (Red) and Elgin (Blue) for the treatments suckered and non-suckered, and north–south and east–west row orientations

The results show the data collected per region and individual treatments on each farm. Data are given in means and bars with same letters show p-value is significant at $p \leq 0.05$ and letters that are not the same show insignificant when $p \geq 0.05$

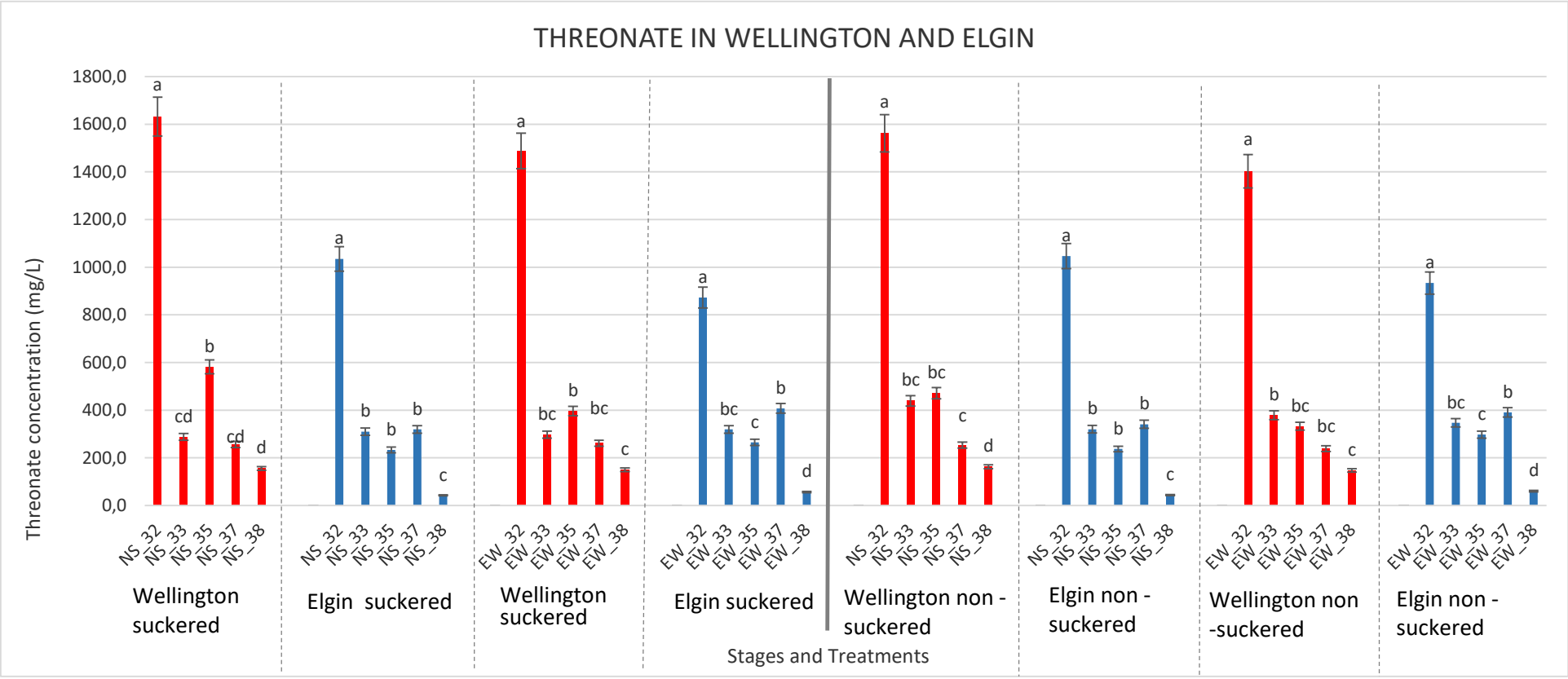

Figure 4.4: L-threonate derivative accumulation between two climatic regions Wellington (Red) and Elgin (Blue) for the treatments suckered and non-suckered, and north–south and east–west row orientations

The results show the data collected per region and individual treatments on each farm. Data are given in means and bars with same letters show p-value is significant at $p \leq 0.05$ and letters that are not the same show insignificant when $p \geq 0.05$

Due to the possible influence of climate differences between the wine grape growing regions of Wellington and Elgin (Figure 4.2–4.4), grape ripening progression and ascorbic acid derivative concentrations varied greatly. It was observed that the ascorbic acid derivative concentrations decreased after E-L 32 for both growing regions (Figure 4.3). In most fruits, such as grapes, acidity decreases at véraison while sugar content increases during ripening (Gautier *et al.*, 2008; Mellidou *et al.,* 2012). The decrease in the ascorbic acid derivative content can be ascribed to abiotic factors such as light intensity, light quality and temperature. These environmental factors then results in the instability of the ascorbic acid and its derivates. Ascorbic acid derivative content of grapes is positively influenced by light intensity during growth (Matei *et al.,* 2009) and negatively influenced by climacteric conditions (rain) (Matei, 2008).

One can observe that the rate of catabolism for ascorbic acid derivatives in grapes is almost unchanged during all the ripening periods from E-L 33 to E-L 37, where it decreases gradually at E-L 38 (Figure 4.2–4.4). That could be because, as the grapes enlarge and accumulate sugar, the total acid concentration in the grapes decreases. There were, however, differences between the two regions in the concentrations of ascorbic acid derivatives during the ripening period.

The treatments of suckered and non-suckered vineyard rows did not show significant differences (Figure 4.2–4.4). The idea behind suckering and leaf removal into the bunch zone was to increase light penetration to the berries. A significant number of studies have investigated the impacts of leaf removal on berry development and ripening (Du Plessis *et al.,* 2017), and that depending on the cultivar, the objectives range from improving the acid balance (De Toda *et al.,* 2013; Baiano *et al.*, 2015; Du Plessis *et al.*, 2017); improving anthocyanin/colour stability (Yu *et al.*, 2016; Pastore *et al.*, 2017); increasing specific secondary metabolites such as volatile aroma precursors (Young *et al.*, 2016) or lowering of metabolites that are perceived negatively in the grapes or wines (Sidhu *et al.,* 2015).

In viticulture, many canopy management practices are performed to optimise light exposure to drive photosynthesis of the canopy (Clingeleffer, 2010). These studies have all highlighted the adaptability of the grapevine berries to the changed microclimate and have also provided scope to investigate mechanisms of perceiving and adapting to the stresses linked to changes in microclimate (Du Plessis *et al.*, 2017). According to Hamner, Bernstein & Maynard (1945), light activates ascorbic acid biosynthesis. In grapes, the ascorbic acid content was twice as high in fruits grown under high light conditions compared to fruits growing in low light (Cruz-Rus *et al.*, 2010). Padayatty *et al.* (2003) reported that areas with cool nights produce citrus fruits with higher ascorbic acid concentrations, and hot tropical

areas produce fruit with lower concentrations of ascorbic acid. In most fruits, acidity decreases while sugar content increases during ripening (Gautier *et al.*, 2008; Mellidou *et al.*, 2012). However, the change in ascorbic acid levels during fruit ripening is a trait dependent on the species. In tomatoes (Badejo *et al.*, 2011), grapes (Cruz-Rus *et al.*, 2010) and strawberries (Cruz-Rus, Amaya, Sanchez-Sevilla, Botella & Valpuesta, 2011), ascorbate content increases as the fruit ripens.

4.4 Principal component analysis (PCA) of Sauvignon blanc wines for Wellington and Elgin

The PCA plot for Wellington and Elgin of the first two discriminant factors (F1, F2) for Sauvignon blanc wines made from the grapes from these two wine regions was done using the data collected from E-L 32 to E-L38 (Figure 4.5). Interestingly the data showed that suckering did not significantly influence the ascorbic acid derivative DHA, DKG and L-threonate concentrations for north–south and east–west rows, and similar trends/behaviours were observed for both Wellington and Elgin regions separately. The grape parameters pH, titratable acidity and the higher concentrations of ascorbic acid derivates corresponded with early ripening stages E-L 32 and E-L 33. The later ripening stages (E-L 35; E-L 37 & E-L 38) corresponded with high sugar content in the grape berries. The row orientations of the vineyard blocks or the treatments applied did not have an impact on the grape parameters.

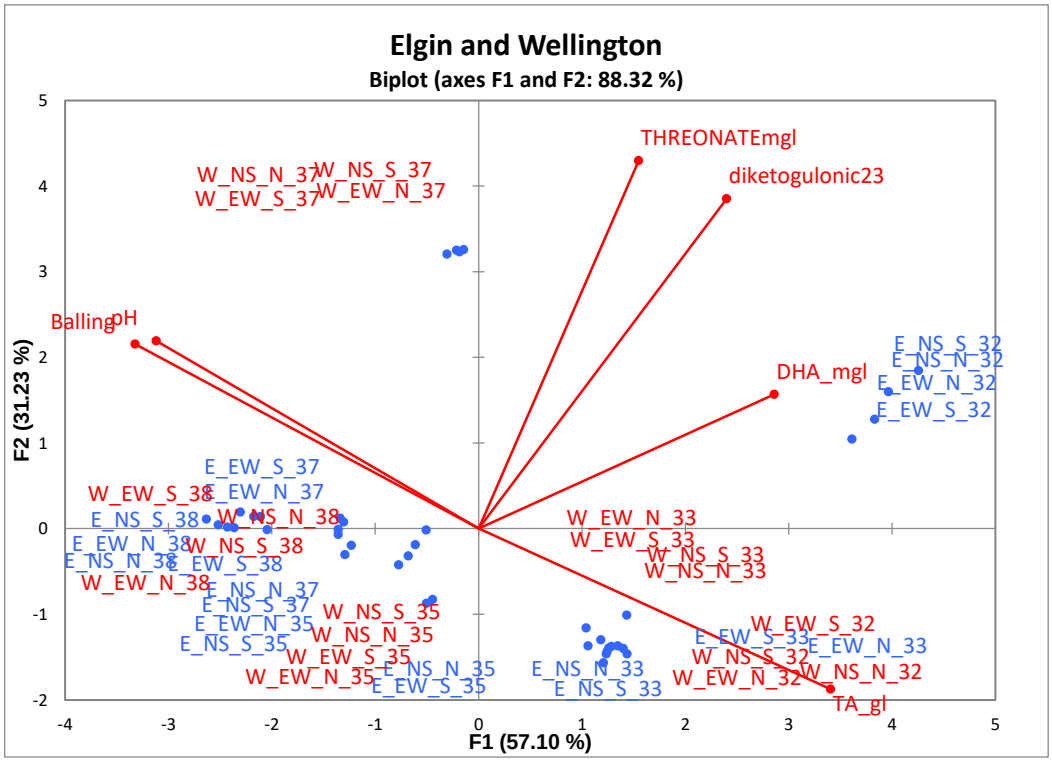

Figure 4.5: PCA biplot of 2018-2019 harvest for Wellington (red marked) and Elgin (blue marked)

It is marked from E-L 32-38 ripening stages, and north–south and east–west suckered and non-suckered rows. Wellington east–west and north–south suckered rows (W_EW_S & W_NS_S); Wellington east–west and north–south non-suckered rows (W_EW_N & W_NS_N). Elgin east–west and north–south suckered rows (E_EW_S & E_NS_S); Elgin east–west and north–south non-suckered rows (E_EW_N & E_NS_N)

When looking at the observations, all the possible treatment combinations from each ripening stage have been grouped. The ripening stage had the most overarching influence on the observed groupings. The total percentage variance was 88.32%. Clear grouping can be observed for the Wellington E-L 37 and Elgin E-L 32 berry growing stages. A progression with ripening can be observed along the F1 axis. DHA, DKG and L-threonate all contribute to the Elgin E-L 32 grouping observed.

4.5 Determination of the pinking phenomenon associated with Sauvignon blanc cultivar between two climates

The pinking potential of the Sauvignon blanc from the two climatic regions of Wellington and Elgin was measured (Figure 4.6). The absorbance values in Figure 4.6 indicates the pinking susceptibility of the two regions. There was a significant difference in the potential to pink between Wellington and Elgin. Sauvignon blanc from Elgin north–south and east–west, suckered and non-suckered rows showed potential to pink. Wellington did not show any pinking potential of the wines. The differences in pinking behaviour between the two regions could be attributed to the fact that cooler grapes can hold more dissolved oxygen than warmer grapes (Nel & Van Jaarsveld, 2018). According to Simpson (1977), the increased

pinking phenomenon in some vintages coincided with an increase in the use of colder fermentation and an inert gas such as nitrogen and carbon dioxide, and closed containers to protect against oxidation. Wines produced under these conditions are often those exhibiting higher susceptibility to pinking when exposed to small amounts of air, often during bottling, and will become brown when further exposed to oxygen at a later stage or when bottled with a high dissolved oxygen content; this is in agreement with observations made by Singleton (1972).

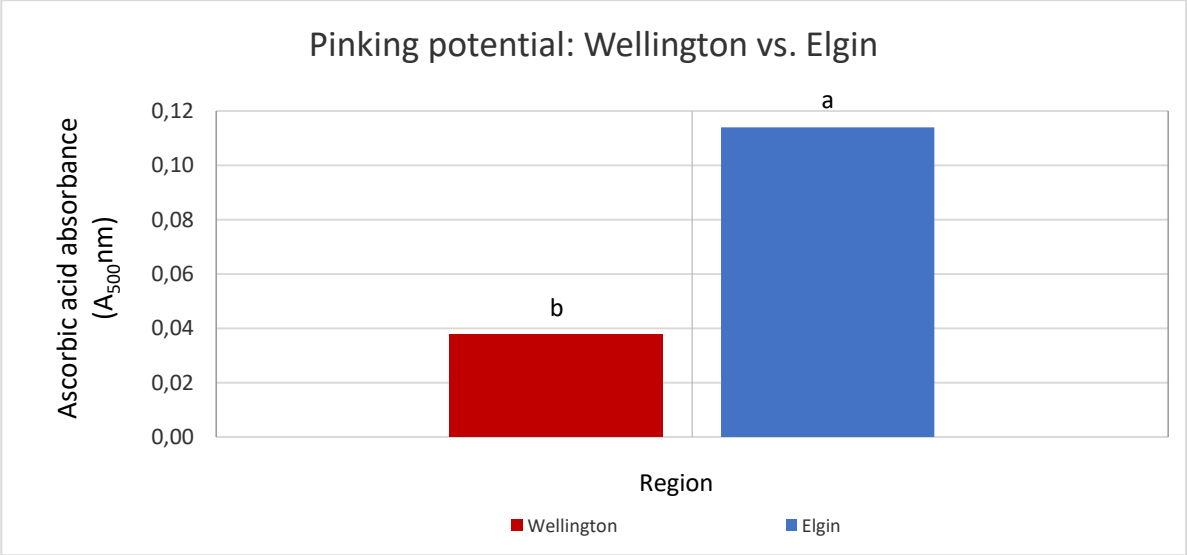

Figure 4.6: Pinking potential of Sauvignon blanc from two climatic regions, Wellington and Elgin

Values are means (*n* = 12) for each farm for all treatments, i.e. north–south and east–west suckered and non-suckered rows. Data are given in means and bars with different letters show a significant difference ($p \leq 0.05$)

Several scientific publications examine possible compounds that promote pinking, but the findings do not all concur. To date, there is uncertainty about which compound causes pinking (Andrea-Silva *et al.*, 2014). Therefore research about pinking is still ongoing. When ascorbic acid (vitamin C) is used, in the presence of oxygen in the juice/wine, the ascorbic acid is oxidised to DHA, ultimately resulting in the formation of hydrogen peroxide (H_2O_2) (Barril *et al*., 2016). H_2O_2, being a strong oxidising agent, requires sulphur dioxide to bind and neutralise the wine/juice (Bradshaw *et al*., 2011). Although ascorbic acid is a very good antioxidant, it has the ability to reduce the shelf life of Sauvignon blanc, and the wines quickly become brown after bottling (Barril *et al.*, 2016). According to Nel and van Jaarsveld (2018), it is important to consider the free SO_2 concentration of the wine at 35–45 mg/L if ascorbic acid is used, and when working with dry ice and inert gas, it is also important to keep the SO_2 of the wine as close as possible to the 35–45 mg/L. Ascorbic acid reacts with SO_2 at a ratio of 1:1.7 and not 1:1 as commonly assumed and it is, therefore, important first to determine the existing concentration of ascorbic acid in the wine before making any ascorbic acid additions/adjustments (Nel & van Jaarsveld, 2018).

4.6 Other ripening grape parameters (pH, titratable acidity (TA) and balling)

The grape berry ripening parameters such as pH, TA and balling was measured during the berry development stages (Table 4.1 & 4.2) (Appendix A-C). During berry development, acidity levels are consistently changing as a result of metabolic activities. During maturation, juice pH increased, and the titratable acids in the juice declined at E-L 32 to E-L 38. The concentration of sugars remained constant for Wellington from E-L 32 to E-L 33 (0.44°B), and it increased at véraison (E-L 35) to harvest (E-L 38). Elgin had an increase in sugar concentration from E-L 32 to E-L 38.

Haggerty (2013) reported that during early development, berries are slow-growing, green, and accumulate high amounts of organic acids but small quantities of sugar. In the second cycle, véraison, berries start softening, colouring, increase in size, sugar content increases and organic acid concentrations decrease (Coombe & McCarthy, 2000; Coombe & Iland, 2005; Haggerty, 2013). Sugar and colour accumulate rapidly in the berry during véraison, with a sharp decline in the total organic acid concentration (Dokoozlian & Kliewer, 1996). The pattern of decline of titratable acidity corresponds with the sharp decline in the malic acid content (Crippen & Morrison, 1986). Tartaric acid is a secondary product formed from the metabolism of glucose and ascorbate (Ruffner, 1982; Haggerty, 2013).

Table 4.1: Means for the Wellington interaction between orientation and stage treatment for the north–south and east–west row orientations

E-L Stage	Treatment	N	pH	TA (g/L)	Balling (°B)
32	N-S	6	2.49 ± 0.03^{d}	37.64 ± 0.58^{a}	0.44 ± 0.004^{d}
	E-W		2.55 ± 0.02^{d}	38.03 ± 0.15^{a}	0.44 ± 0.004^{d}
33	N-S	6	2.49 ± 0.03^{d}	32.32 ± 0.80^{b}	0.44 ± 0.004^{d}
	E-W		2.55 ± 0.02^{d}	33.11 ± 0.90^{b}	0.44 ± 0.004^{d}
35	N-S	6	2.68 ± 0.01^{c}	17.00 ± 0.30^{c}	16.57 ± 0.27^{c}
	E-W		2.68 ± 0.02^{c}	16.97 ± 0.26^{c}	16.72 ± 0.16^{c}
37	N-S	6	3.00 ± 0.02^{b}	12.11 ± 0.43^{d}	19.93 ± 0.25^{b}
	E-W		2.98 ± 0.21^{b}	11.65 ± 0.30^{d}	20.03 ± 0.23^{b}
38	N-S	6	3.07 ± 0.01^{a}	7.30 ± 0.20^{e}	21.39 ± 0.85^{a}
	E-W		3.07 ± 0.04^{a}	7.49 ± 1.20^{e}	21.19 ± 0.58^{a}
Least Significant Difference ($LSD_{0.05}$)	N-S		0.035	0.675	0.228
	E-W		0.024	0.563	0.134

Table 4.2: Means for the Elgin interaction between orientation and stage treatment for the north–south and east–west row directions

E-L Stage	Treatment	N	pH	TA (g/L)	Balling (°B)
32	N-S	6	2.52 ± 0.01^{c}	42.08 ± 0.79^{a}	0.42 ± 0.004^{d}
	E-W		2.53 ± 0.02^{c}	41.25 ± 0.48^{a}	0.42 ± 0.01^{d}
33	N-S	6	2.52 ± 0.02^{c}	37.23 ± 0.70^{b}	0.63 ± 0.02^{d}
	E-W		2.52 ± 0.01^{c}	36.52 ± 0.38^{b}	0.62 ± 0.01^{d}
35	N-S	6	2.87 ± 0.54^{ab}	24.95 ± 0.45^{c}	16.27 ± 0.37^{c}
	E-W		2.87 ± 0.54^{ab}	25.26 ± 0.37^{c}	16.25 ± 0.36^{c}
37	N-S	6	2.83 ± 0.01^{b}	10.68 ± 0.22^{d}	18.90 ± 0.19^{b}
	E-W		2.83 ± 0.01^{b}	10.63 ± 0.26^{d}	19.00 ± 0.22^{b}
38	N-S	6	3.16 ± 0.01^{a}	7.66 ± 0.25^{e}	23.74 ± 0.44^{a}
	E-W		3.14 ± 0.02^{a}	8.15 ± 0.14^{e}	21.67 ± 0.41^{a}
Least Significant Difference ($LSD_{0.05}$)	N-S		0.29	0.69	0.25
	E-W		0.29	0.47	0.34

Most tartaric acid is formed pre-veraison but by different biosynthetic and metabolic pathways. Grapes are one of the only fruits containing tartaric acid, and for most *Vitis vinifera* cultivars, it is the predominant acid (Shiraishi, 1995; Liu *et al.,* 2006). The concentration of tartaric acid decreases after veraison due to dilution from the influx of sugar and water to the berry (Haggerty, 2013). However, unlike malic acid, there is minimal degradation, and only a trace amount of tartaric acid is metabolised through respiration (Ruffner, 1982). The reduction in TA during fruit ripening is partly related to the respiration of malic acid in the berry and is, therefore, related to temperature (Gladstones, 1992). Grapes grown in warmer regions (higher heat summation units) mature earlier and have a lower TA at the same soluble solids concentration when compared to fruit grown in a cooler climate (Gladstones, 1992). A characteristic of cooler growing regions is lower daily temperature fluctuations during the late stages of fruit ripening, an important contributor to acid retention (Gladstones, 1992). Enhanced knowledge of the range of variation for acids and sugars throughout berry development will guide grape growers in harvest decisions and winemakers in their winemaking process.

CHAPTER 5:
CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The overall aim of the study was to monitor ascorbic acid derivative levels in Sauvignon blanc (*Vitis vinifera* L.) during berry development and in wine in South Africa. Analysis of ascorbic acid derivatives across physiological stages of fruit development was a key aspect of this research, given the link between ascorbic acid metabolism and its precursors. The concentrations of ascorbic acid derivatives were measured for both farms on a north–south and east–west vineyard orientation on suckered and non-suckered vineyard rows. The results showed that there were no significant differences between the suckered and non-suckered vineyard rows and that the vineyard rows did not show any impact on the accumulation of ascorbic acid derivatives. Although they were no significant differences between the vineyard blocks on each farm (Wellington & Elgin), there were significant differences of ascorbic acid derivatives between the two farms. The differences may be due to the climatic conditions (meso, micro and macro-climate) in which the grapevines are exposed to on each farm.

Results showed that the accumulation of ascorbic acid derivatives varies between regions because of different climatic conditions. The conclusion that was drawn was that there is close regulation on ascorbic acid biosynthesis and the concentration in plant tissues is a result of balancing synthesis with the catabolism of ascorbic acid. Catabolism would be the result of a breakdown of ascorbic acid to DHA, DKG, L-threonate and other compounds. In biological systems, ascorbic acid can be found only at low pH, but in neutral solutions above pH 5, it is predominantly found in the ionized form, ascorbate, and this would deplete the total ascorbic acid concentration. The concentrations are high at E-L 32 and subsequently decrease as the grapes ripen. Interestingly the threonic acid, one of the derivatives, was predominant in almost all the stages of berry development, other derivatives DHA and DKG were fluctuating between the stages. The ascorbic acid derivatives accumulation were affected by the area in which the vines were planted.

The pinking potential of the wines, on the other hand, showed clear differences between regions, with wines vinified from Elgin grapes highly susceptible to pinking. Advancements in the field of ascorbic acid research have been accelerated by consumer interest in the nutritional value of this antioxidant. It is important to understand the mechanisms that determine the levels of ascorbic acid derivatives in fruits, a major source for ascorbic acid in the human diet.

In the present study, the accumulation patterns of ascorbic acid derivatives were closely correlated to each other as shown by the PCA biplot grouping for Elgin and Wellington. Ascorbic acid is known to be beneficial in protecting white wines against pinking, and may well contribute to the delay of atypical ageing (ATA). Research has now shown that the addition of ascorbic acid reduces the amount of brown colour and oxidised character produced in some wine during bottle maturation (Barril *et al.*, 2016).

The continued existence and welfare of the producer is directly dependent on the sustainable profitability of the wine industry. In contrast, the increasingly health-conscious and environmentally conscious wine consumer is looking for Individualised products with a high ratio of quality to price. With all the competition in the wine industry, producers can use less SO_2 in correlation with ascorbic acid in their wines and on the other side, satisfying consumer needs that are allergic to sulphur compounds. Due to the above-mentioned importance, knowledge regarding the development, maturation and vitamin contents of grape berries is of great economic interest to increase the number of health benefits attributed to grapes and wine.

5.2 Recommendations and future research

Ascorbic acid reacts spontaneously with dissolved oxygen to yield its derivatives, thus decreasing in concentration. As a result, its use in wine requires some careful precautions. The study showed that care should be taken when working with ascorbic acid, as it easily oxidises to other forms. Considering that specific environmental stimuli can influence the accumulation of ascorbic acid and derivatives, changes to current vineyard management systems may be sufficient to regulate the ascorbic acid metabolism of berries without resorting to genetic strategies. It is, therefore, recommended that more needs to be done on linking the ascorbic acid derivatives with the grape clones that are available in the industry, as clones could affect the synthesis of ascorbic acid. Researchers can use this study as a model for designing experiments to identify factors that influence the stability of ascorbic acid and other compounds during grape processing. The shortcoming of the study was the sampling procedure in which the berries were pulled off from the pedicel. Future studies should focus more on cutting the berries with the pedicel to prevent berry juice oxidation. Several attempts were made to identify the peaks of ascorbic acid by liquid chromatography-mass spectrometry (LC/MS) but without success. The only useful data obtained indicated that the main peaks of ascorbic acid derivatives. LC/MS has its limitations when applied to small molecules and unfortunately it did not yield any useful information in this instance. To identify ascorbic acid and the degradation products for future research, it will be necessary to use other techniques more suited to small molecule analysis.

Due to increased wine consumption, more focused studies should be conducted on the effects climate and other factors have on the preservation of wine grapes derived ascorbic acid. These studies will assist us in understanding the influence of climate on ascorbic acid metabolism in grapevines, by using artificial shading (greenhouses) experiments in controlled environments.

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APPENDICES

Appendix A
Analysis of variance (ANOVA)

When the data was not normally distributed or the Levene's test for homogenous of variance was violated, the non-parametric Mann-Whitney-U test was used, and it revealed the same results as the independent sample t-test.

P-values for ANOVA for Wellington - all data

Source	DF	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
Direction	1	0.0006	0.3409	0.0141	0.0011	0.1873	0.8390
Treatment	1	0.9303	0.1949	0.5799	0.1683	0.2170	0.0049
Treatment x Direction	1	0.2258	0.9090	0.4542	0.4394	0.5535	0.0763
Stage	4	0.0004	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment x Stage	4	0.6830	0.3869	0.1097	0.0638	0.0055	0.0001
Direction x Stage	4	0.0008	0.0250	0.1612	0.0002	0.0622	0.0822
Direction x Treatment x Stage	4	0.8588	0.9981	0.9747	0.0233	0.0069	0.0001

Table of means for the Wellington interaction between treatment and stage for the East to West row directions

E-L Stage	N	Treatment	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
32	3	Non-Suckered	28.85 ± 5.95^{b}	636.85 ± 25.94^{a}	1402.35 ± 108.58^{a}	2.56 ± 0.01^{e}	38.11 ± 0.18^{a}	0.44 ± 0.00^{f}
33	3	Non-Suckered	27.70 ± 3.99^{b}	116.92 ± 29.81^{b}	378.76 ± 258.77^{b}	2.56 ± 0.01^{e}	32.62 ± 0.97^{c}	0.44 ± 0.00^{f}
35	3	Non-Suckered	25.42 ± 10.14^{b}	30.74 ± 5.84^{c}	333.04 ± 41.39^{bc}	2.69 ± 0.02^{d}	16.93 ± 0.30^{d}	16.63 ± 0.05^{e}
37	3	Non-Suckered	47.66 ± 3.11^{a}	143.34 ± 19.34^{d}	238.35 ± 17.69^{bc}	2.98 ± 0.01^{c}	11.62 ± 0.28^{e}	20.18 ± 0.19^{c}
38	3	Non-Suckered	24.73 ± 4.73^{b}	8.66 ± 1.20^{e}	147.13 ± 21.33^{c}	3.03 ± 0.01^{b}	8.59 ± 0.03^{f}	20.67 ± 0.02^{b}
32	3	Suckered	27.60 ± 8.18^{b}	580.91 ± 32.23^{a}	1488.26 ± 122.64^{a}	2.54 ± 0.03^{e}	37.96 ± 0.11^{a}	0.44 ± 0.00^{f}
33	3	Suckered	28.74 ± 8.27^{b}	94.72 ± 24.81^{b}	297.60 ± 46.38^{bc}	2.54 ± 0.03^{e}	33.60 ± 0.63^{b}	0.44 ± 0.00^{f}
35	3	Suckered	30.67 ± 1.03^{b}	39.82 ± 4.80^{c}	396.64 ± 40.59^{b}	2.67 ± 0.03^{d}	17.02 ± 0.27^{d}	16.82 ± 0.19^{d}
37	3	Suckered	52.25 ± 9.93^{a}	141.33 ± 16.90^{d}	261.20 ± 21.20^{bc}	2.98 ± 0.03^{c}	11.69 ± 0.39^{e}	19.88 ± 0.18^{d}

38	3	Suckered	32.53 ± 1.77^{b}	8.66 ± 1.10^{e}	150.26 ± 5.69^{c}	3.12 ± 0.01^{a}	6.39 ± 0.14^{g}	21.72 ± 0.08^{a}
Least Significant Difference (LSD)			10.702	37.629	90.408	0.034	0.797	0.190

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different

Table of means for the Wellington interaction between treatment and stage for the North to South row directions

E-L Stage	**N**	**Treatment**	**DHA**	**DKG**	**L-threonate**	**pH**	**TA (g/L)**	**Balling (°B)**
32	3	Non-Suckered	25.45 ± 1.54^{abcd}	544.36 ± 78.49^{a}	1562.01 ± 166.73^{a}	2.47 ± 0.01^{d}	37.63 ± 0.32^{a}	0.45 ± 0.00^{d}
33	3	Non-Suckered	68.56 ± 35.52^{a}	152.75 ± 59.51^{b}	439.45 ± 99.06^{bc}	2.47 ± 0.01^{d}	32.51 ± 0.87^{b}	0.45 ± 0.00^{d}
35	3	Non-Suckered	46.65 ± 9.98^{abc}	34.23 ± 3.36^{c}	471.70 ± 15.58^{bc}	2.68 ± 0.02^{c}	16.75 ± 0.19^{c}	16.57 ± 0.43^{c}
37	3	Non-Suckered	45.57 ± 5.95^{abcde}	145.16 ± 11.39^{b}	253.38 ± 4.95^{c}	3.00 ± 0.03^{b}	12.28 ± 0.52^{d}	19.87 ± 0.26^{b}
38	3	Non-Suckered	44.83 ± 4.57^{abcd}	9.93 ± 0.98^{c}	162.70 ± 2.26^{d}	3.07 ± 0.01^{a}	7.42 ± 0.22^{e}	21.35 ± 0.04^{a}
32	3	Suckered	19.12 ± 2.82^{abcd}	486.67 ± 46.43^{a}	1631.72 ± 145.61^{a}	2.50 ± 0.05^{d}	37.66 ± 0.87^{a}	0.44 ± 0.01^{d}
33	3	Suckered	54.36 ± 34.51^{ab}	114.88 ± 15.63^{bc}	287.88 ± 163.03^{cd}	2.51 ± 0.05^{d}	32.14 ± 0.86^{b}	0.44 ± 0.01^{d}
35	3	Suckered	54.56 ± 21.45^{ab}	37.43 ± 2.21^{c}	581.56 ± 59.02^{b}	2.68 ± 0.02^{c}	17.24 ± 0.14^{c}	16.59 ± 0.02^{c}
37	3	Suckered	45.68 ± 5.63^{d}	152.82 ± 11.46^{b}	256.30 ± 30.64^{cd}	3.00 ± 0.03^{b}	11.94 ± 0.31^{d}	20.00 ± 0.28^{b}
38	3	Suckered	37.23 ± 3.42^{bcd}	10.00 ± 0.96^{c}	155.76 ± 6.26^{d}	3.08 ± 0.01^{a}	7.18 ± 0.10^{e}	21.44 ± 0.09^{a}
Least Significant Difference (LSD $_{0.05}$)			26.756	104.96	167.18	0.05	0.954	0.323

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different

Table of means for the Wellington interaction between direction and stage treatment for the North to South and East to West row directions

E-L Stage	N	Row Direction	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
32	6	N-S	22.28 ± 4.02^{c}	515.52 ± 65.76^{a}	1597.17 ± 145.95^{a}	2.49 ± 0.03^{d}	37.64 ± 0.58^{a}	0.44 ± 0.004^{d}
		E-W	28.22 ± 6.43^{b}	608.88 ± 40.29^{a}	1445.31 ± 113.78^{a}	2.55 ± 0.02^{d}	38.03 ± 0.15^{a}	0.44 ± 0.004^{d}
33	6	N-S	61.46 ± 32.27^{a}	133.82 ± 100.19^{b}	348.51 ± 150.44^{c}	2.49 ± 0.03^{d}	32.32 ± 0.80^{b}	0.44 ± 0.004^{d}
		E-W	28.22 ± 5.83^{b}	105.82 ± 27.38^{c}	346.30 ± 189.72^{b}	2.55 ± 0.02^{d}	33.11 ± 0.90^{b}	0.44 ± 0.004^{d}
35	6	N-S	50.61 ± 15.58^{ab}	35.83 ± 3.09^{c}	526.71 ± 71.49^{b}	2.68 ± 0.01^{c}	17.00 ± 0.30^{c}	16.57 ± 0.27^{c}
		E-W	28.05 ± 7.06^{b}	35.28 ± 6.89^{d}	364.84 ± 50.57^{b}	2.68 ± 0.02^{c}	16.97 ± 0.26^{c}	16.72 ± 0.16^{c}
37	6	N-S	45.63 ± 5.18^{ab}	148.99 ± 11.05^{b}	254.86 ± 19.69^{cd}	3.00 ± 0.02^{b}	12.11 ± 0.43^{d}	19.93 ± 0.25^{b}
		E-W	49.96 ± 7.18^{a}	142.33 ± 16.28^{b}	249.77 ± 21.60^{bc}	2.98 ± 0.21^{b}	11.65 ± 0.30^{d}	20.03 ± 0.23^{b}
38	6	N-S	41.03 ± 5.51^{bc}	9.96 ± 0.87^{c}	159.23 ± 5.67^{d}	3.07 ± 0.01^{a}	7.30 ± 0.20^{e}	21.39 ± 0.85^{a}
		E-W	28.63 ± 5.33^{b}	8.66 ± 1.03^{e}	148.70 ± 14.07^{c}	3.07 ± 0.04^{a}	7.49 ± 1.20^{e}	21.19 ± 0.58^{a}
Least Significant Difference ($LSD_{0.05}$)			N-S= 18.919; E-W= 7.567	N-S= 74.21; E-W= 26.608	E-W= 133.11; N-S= 117.65	N-S= 0.035; E-W= 0.024	N-S= 0.675; E-W= 0.563	N-S= 0.228; E-W= 0.134

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different

Appendix B

P-values for ANOVA for Elgin - all data

Source	DF	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
Direction	1	0.8262	0.2085	0.8955	0.9634	0.2160	0.0001
Treatment	1	0.8728	0.8374	0.3613	0.9746	0.8700	0.1281
Treatment x Direction	1	0.5129	0.4680	0.7378	0.1788	0.3033	0.3719
Stage	4	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment x Stage	4	0.9998	0.9442	0.9661	0.9998	0.9101	0.1010
Direction x Stage	4	0.9840	0.0047	0.0049	1.0000	0.0049	0.0001
Direction x Treatment x Stage	4	0.8093	0.5134	0.9445	0.1031	0.2473	0.2309

Table of means for the Elgin interaction between treatment and stage for the East to West row directions

E-L Stage	N	Treatment	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
32	3	Non-Suckered	460.68 ± 82.57^{a}	551.66 ± 133.21^{a}	933.70 ± 117.28^{a}	2.52 ± 0.00b	41.54 ± 0.31^{a}	0.42 ± 0.01^{d}
33	3	Non-Suckered	51.15 ± 2.77^{b}	77.53 ± 6.67^{b}	347.00 ± 8.24^{bc}	2.52 ± 0.00b	36.43 ± 0.55^{b}	0.63 ± 0.00^{d}
35	3	Non-Suckered	18.43 ± 7.67^{b}	17.16 ± 3.24^{b}	297.37 ± 52.54^{c}	2.66 ± 0.01b	25.33 ± 0.31^{c}	16.23 ± 0.45^{c}
37	3	Non-Suckered	21.49 ± 1.16^{b}	53.14 ± 7.18^{b}	391.01 ± 15.34^{b}	3.10 ± 0.76ab	10.68 ± 0.31d	18.35 ± 0.05^{b}
38	3	Non-Suckered	13.04 ± 0.11^{b}	10.45 ± 2.53^{b}	60.04 ± 6.36^{d}	3.13 ± 0.01^{a}	8.20 ± 0.12^{e}	21.61 ± 0.48^{a}
32	3	Suckered	425.24 ± 87.92^{a}	516.64 ± 104.56^{a}	873.04 ± 118.36^{a}	2.53 ± 0.02^{b}	40.96 ± 0.46^{a}	0.41 ± 0.00^{d}
33	3	Suckered	48.35 ± 4.99^{b}	81.76 ± 10.42^{b}	319.45 ± 24.01^{bc}	2.53 ± 0.01^{b}	36.60 ± 0.20b	0.61 ± 0.01^{d}
35	3	Suckered	15.55 ± 1.13^{b}	15.20 ± 3.94^{b}	264.96 ± 34.51^{c}	2.64 ± 0.00^{b}	25.19 ± 0.49^{c}	16.26 ± 0.35^{c}
37	3	Suckered	20.27 ± 4.90^{b}	51.73 ± 6.88^{b}	407.77 ± 43.44^{b}	2.83 ± 0.01^{ab}	10.57 ± 0.25^{d}	19.04 ± 0.34^{b}
38	3	Suckered	9.39 ± 1.69^{b}	6.53 ± 0.78^{b}	56.78 ± 5.04^{d}	3.15 ±0.02^{a}	8.10 ± 0.16^{e}	21.72 ± 0.42^{a}
Least Significant Difference ($LSD_{0.05}$)			66.92	90.318	91.524	0.419	0.658	0.482

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different

Table of means for the Elgin interaction between treatment and stage for the North to South row directions

E-L Stage	N	Treatment	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
32	3	Non-Suckered	440.83 ± 143.18^{a}	630.34 ± 137.26^{a}	1047.05 ± 211.20^{a}	2.52 ± 0.00^{b}	41.68 ± 1.05^{a}	0.42 ± 0.00^{e}
33	3	Non-Suckered	52.24 ± 1.45^{b}	65.21 ± 4.11^{b}	320.24 ± 18.14^{b}	2.53 ± 0.02^{b}	37.26 ± 1.08^{b}	0.64 ± 0.00^{e}
35	3	Non-Suckered	12.67 ± 4.70^{b}	8.47 ± 1.12^{b}	237.06 ± 11.49^{b}	2.65 ± 0.01^{b}	24.91 ± 0.45^{c}	16.22 ± 0.29^{d}
37	3	Non-Suckered	25.35 ± 5.06^{b}	40.14 ± 5.64^{b}	340.96 ± 49.54^{b}	2.83 ± 0.01^{ab}	10.60 ± 0.24^{d}	18.95 ± 0.26^{c}
38	3	Non-Suckered	9.11 ± 0.65^{b}	4.84 ± 1.86^{b}	43.96 ± 4.50^{c}	3.14 ± 0.01^{a}	7.86 ± 0.11^{e}	23.33 ± 0.04^{a}
32	3	Suckered	475.86 ± 36.52^{a}	720.88 ± 128.75^{a}	1034.57 ± 115.67^{a}	2.51 ± 0.01^{b}	42.46 ± 0.14^{a}	0.41 ± 0.00^{e}

33	3	Suckered	43.70 ± 5.19[b]	46.50 ± 1.56[b]	309.75 ± 28.16[b]	2.51 ± 0.00[b]	37.21 ± 0.25[b]	0.61 ± 0.01[e]
35	3	Suckered	17.25 ± 4.90[b]	9.58 ± 0.13[b]	233.34 ± 7.08[b]	2.82 ± 0.01[a]	24.98 ± 0.54[c]	16.33 ± 0.50[d]
37	3	Suckered	23.34 ± 1.12[b]	36.37 ± 4.65[b]	319.40 ± 41.95[b]	3.09 ± 0.76[ab]	10.76 ± 0.19[d]	18.84 ± 0.09[c]
38	3	Suckered	8.00 ± 0.39[b]	4.22 ± 0.52[b]	42.35 ± 0.23[c]	3.17 ± 0.01[a]	7.45 ± 0.11[e]	24.14 ± 0.02[b]
Least Significant Difference ($LSD_{0.05}$)			82.178	102.87	132.07	0.4174	0.9001	0.3575

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different

Table of means for the Elgin interaction between direction and stage treatment for North to South and East to West row directions

E-L Stage	N	Row Direction	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
32	6	N-S	458.95 ± 95.41[a]	675.92 ± 128.82[a]	1040.82 ± 152.45[a]	2.52 ± 0.01[c]	42.08 ± 0.79[a]	0.42 ± 0.004[d]
		E-W	442.96 ± 78.71[a]	534.15 ± 108.81[a]	903.37 ± 110.49[a]	2.53 ± 0.02[c]	41.25 ± 0.48[a]	0.42 ± 0.01[d]
33	6	N-S	47.97 ± 5.78[b]	55.86 ± 10.62[b]	314.99 ± 21.95[bc]	2.52 ± 0.02[c]	37.23 ± 0.70[b]	0.63 ± 0.02[d]
		E-W	49.75 ± 3.92[b]	79.64 ± 8.16[b]	333.23 ± 22.03[c]	2.52 ± 0.01[c]	36.52 ± 0.38[b]	0.62 ± 0.01[d]
35	6	N-S	14.96 ± 4.98[b]	9.03 ± 0.94[b]	235.20 ± 8.77[c]	2.87 ± 0.54[ab]	24.95 ± 0.45[c]	16.27 ± 0.37[c]
		E-W	16.99 ± 5.15[b]	16.18 ± 3.4 [bc]	281.17 ± 43.54[c]	2.87 ± 0.54[ab]	25.26 ± 0.37[c]	16.25 ± 0.36[c]
37	6	N-S	24.34 ± 3.46[b]	38.26 ± 5.06[b]	330.19 ± 42.72[b]	2.83 ± 0.01[b]	10.68 ± 0.22[d]	18.90 ± 0.19[b]
		E-W	20.88 ± 3.25[b]	52.44 ± 6.34[bc]	399.39 ± 30.55[b]	2.83 ± 0.01[b]	10.63 ± 0.26[d]	19.00 ± 0.22[b]
38	6	N-S	8.56 ± 0.78[b]	4.53 ± 1.27[b]	43.16 ± 2.99[d]	3.16 ± 0.01[a]	7.66 ± 0.25[e]	23.74 ± 0.44[a]
		E-W	11.21 ± 2.27[b]	8.49 ± 2.72[c]	58.41 ± 5.44[d]	3.14 ± 0.02[a]	8.15 ± 0.14[e]	21.67 ± 0.41[a]
Least Significant Difference ($LSD_{0.05}$)			N-S = 58.11; E-W = 47.32	N-S = 72.73; E-W = 63.86	N-S = 93.38; E-W = 64.72	N-S = 0.29; E-W = 0.29	N-S = 0.69; E-W = 0.47	N-S = 0.25; E-W = 0.34

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different

Appendix C

P-values for Wellington ANOVA row directions combined by farm

Source	DF	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
Direction	1	0.0010	0.2283	0.0001	0.0011	0.7427	0.1576
Treatment	1	0.9218	0.1397	0.4478	0.1683	0.0001	0.0038
Treatment x Direction	1	0.0961	0.2087	0.4337	0.4394	0.0012	0.0129
Stage	4	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment x Stage	4	0.5097	0.0010	0.0001	0.0638	0.0001	0.0236
Direction x Stage	4	0.0008	0.0081	0.0003	0.0002	0.0260	0.0491
Direction x Treatment x Stage	4	0.9149	0.0655	0.4883	0.0233	0.0001	0.0149

P-values for Wellington and Elgin ANOVA row directions combined by farm for all treatments

Source	DF	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
Direction	1	0.9693	0.0001	0.0001	0.4056	0.2013	0.0010
Treatment	1	0.0323	0.0001	0.0970	0.5705	0.2619	0.2881
Treatment x Direction	1	0.7307	0.1594	0.6124	0.2742	0.6912	0.7900
Stage	4	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Treatment x Stage	4	0.6017	0.0044	0.9686	0.0178	0.1175	0.0234
Direction x Stage	4	0.1754	0.0001	0.0001	0.1919	0.0001	0.0001
Direction x Treatment x Stage	4	0.8080	0.0812	0.7609	0.4431	0.1319	0.6442

Table of means for the Wellington and Elgin combined data

E-L Stage	N	Farm	DHA	DKG	L-threonate	pH	TA (g/L)	Balling (°B)
32	12	Wellington	3.86 ± 0.13^{a}	4.98 ± 0.09^{b}	5.53 ± 0.08^{d}	2.52 ± 0.04^{d}	3.63 ± 0.01^{a}	-0.81 ± 0.01^{d}
		Elgin	6.09 ± 0.20^{a}	6.38 ± 0.23^{a}	6.87 ± 0.14^{a}	2.54 ± 0.01^{d}	3.72 ± 0.02^{a}	-0.86 ± 0.02^{e}
33	12	Wellington	3.64 ± 0.57^{ab}	4.57 ± 0.35^{c}	5.82 ± 0.28^{c}	2.52 ± 0.04^{d}	3.48 ± 0.03^{b}	-0.81 ± 0.01^{d}
		Elgin	3.88 ±0.10^{b}	4.19 ± 0.24^{b}	5.77 ± 0.07^{b}	2.52 ± 0.01^{d}	3.61 ± 0.02^{b}	-0.47 ± 0.03^{d}
35	12	Wellington	3.59 ± 0.42^{b}	3.56 ± 0.15^{d}	6.08 ± 0.23^{b}	2.68 ± 0.02^{c}	2.83 ± 0.02^{c}	2.81 ± 0.01^{c}
		Elgin	2.78 ± 0.26^{c}	2.48 ± 0.33^{c}	5.54 ± 0.14^{c}	2.65 ± 0.01^{c}	3.22 ± 0.02^{c}	2.79 ± 0.02^{c}
37	12	Wellington	3.20 ± 0.23^{c}	6.32 ± 0.13^{a}	7.32 ± 0.09^{a}	2.99 ± 0.03^{b}	2.47 ± 0.04^{d}	2.99 ± 0.01^{b}
		Elgin	3.10 ± 0.16^{d}	3.79 ± 0.20^{d}	5.89 ± 0.14^{d}	2.83 ± 0.01^{b}	2.37 ± 0.02^{d}	2.94 ± 0.01^{b}
38	12	Wellington	3.52 ± 0.25^{b}	2.22 ± 0.12^{e}	5.03 ± 0.08^{e}	3.07 ± 0.03^{a}	1.99 ± 0.11^{e}	3.06 ± 0.01^{a}
		Elgin	2.27 ± 0.20^{e}	1.86 ± 0.37^{e}	3.91 ± 0.18^{e}	3.15 ± 0.02^{a}	2.07 ± 0.04^{e}	3.12 ± 0.05^{a}
Least Significant Difference ($LSD_{0.05}$)			Wel = 0.226; EL = 0.1665	Wel = 0.110; EL = 0.1239	Wel = 0.086; EL = 0.0805	Wel = 0.020; EL = 0.0106	Wel = 0.019; EL = 0.0154	Wel = 0.009; EL = 0.0164

All values displayed in table are the averages, with the standard deviation expressed after '±'. Means with the same letter are not significantly different