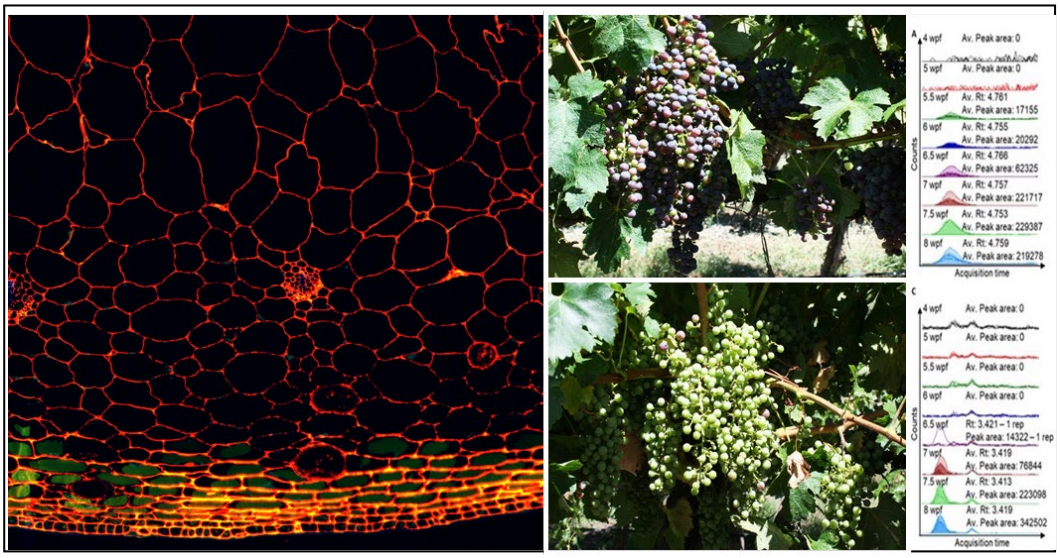
Australian Government

Wine Australia

CSIRO

Adjusting grape berry ripening to suit a changing climate: plant growth regulator-based solutions



FINAL REPORT TO WINE AUSTRALIA

Project Number: **CSA 1701-2.2**

Principal Investigator: **Christopher Davies**

Research Organisation: **CSIRO Agriculture and Food**

Date: **26 August 2022**

Project title: Adjusting grape berry ripening to suit a changing climate: plant growth regulator-based solutions

Author details:
Dr Christopher Davies
Team Leader
CSIRO Agriculture and Food
PO Box 350
Glen Osmond,
SA 5064
Australia

Tel: +61 8 83038628
Email: christopher.davies@csiro.au

Dr Christine Böttcher
Senior Research Scientist
CSIRO Agriculture and Food
PO Box 350
Glen Osmond,
SA 5064
Australia

Tel: +61 8 8273 8126
Email: christine.bottcher@csiro.au

Date report completed: 20 July 2022

Publisher: CSIRO

Copyright: © 2022 CSIRO. To the extent permitted by law, all rights are reserved and no part of this publication covered by copyright may be reproduced or copied in any form or by any means without the written permission of CSIRO.

Important Disclaimer: CSIRO advises that the information contained in this publication comprises general statements based on scientific research. The reader is advised and needs to be aware that such information may be incomplete or unable to be used in any specific situation. No reliance or actions must therefore be made on that information without seeking prior expert professional, scientific and technical advice. To the extent permitted by law, CSIRO (including its employees and consultants) exclude all liability for any consequences, including, but not limited to all losses, damages, costs, expenses and any other compensation, arising directly or indirectly from using this publication (in part or in whole) and any information or materials contained in it.

Table of Contents

1. Abstract 4

2. Executive summary 5

3. Background 8

4. Project aims 11

5. Method 15

5.1 Spray treatments 15

5.2 Basic berry measurements 15

5.3 Analysis of plant macro- and micronutrients 15

5.4 Amino acid analysis 15

5.5 Small-scale winemaking 16

5.6 Laboratory-scale winemaking and headspace volatile analysis 16

5.7 Quantification of methoxypyrazines and rotundone in grape tissue 16

5.8 Analysis of headspace volatiles 16

5.9 RNA extraction, cDNA synthesis and qRT-PCR 16

5.10 Quantification of NAA in grapes 16

5.11 Quantification of NAA in wine 17

5.12 Data analysis 17

6. Results and discussion 18

6.1 The testing of multiple PGRs to alter veraison/harvest date in Cabernet Sauvignon and Shiraz 18

6.2 Use of auxin to delay ripening – sensory and biochemical evaluation of Cabernet Sauvignon and Shiraz 40

6.3 Determination of the effect of auxin application timing on its effectiveness as a delayer of ripening 56

6.4 Auxin treatment of grapevine (*Vitis vinifera L.*) berries delays ripening onset by inhibiting cell expansion 76

6.5 Testing low range NAA application rates for effectiveness in delaying Cabernet Sauvignon veraison/harvest 96

6.6 Ripening delay resulting from NAA application is NAA concentration dependent 101

6.7 Testing for possible interactions between NAA and two commonly used fungicides and an insecticide that might impact on NAA efficacy in delaying ripening 106

6.8 Testing for possible interactions between NAA and four wetting agents with different chemistry - impact on NAA efficacy in delaying ripening and NAA residue levels 109

6.9 Testing the effect of complete vine spray vs bunch-targeted sprays on vine health and on the length of delay in ripening 113

6.10 Determining the location of NAA residue in treated grape berries 121

6.11 Residues in NAA-treated fruit and wines – NAA decay over time 123

6.12 Identification of non-volatile marker compounds for determining the most efficacious time for NAA treatment to delay berry ripening/harvest 125

6.13 Peduncle-girdling of Shiraz (*Vitis vinifera* L.) bunches alters amino acid and hormone content of grapes and affects wine volatile compounds depending on berry sugar levels 152

6.14 Post-veraison restriction of phloem import into Riesling (*Vitis vinifera* L.) berries induces transient and stable changes to fermentation-derived and varietal wine volatiles 170

6.15 Partial girdling as a tool to study the relationship between sugar accumulation, time-on-vine and wine composition 177

6.16 Phenotyping the CSIRO First Generation mildew resistant lines and SARDI wine grape varietal collection for phenological and agronomic traits 186

6.17 Testing of various agents with the potential to alter budburst timing, in-field experiments 201

7. Outcomes and conclusions 204

8. Recommendations 205

Appendix 1: Communication 206

Appendix 2: Intellectual property 208

Appendix 3: References 209

Appendix 4: Staff 224

Appendix 5: Supplemental material 225

Appendix 6: Budget reconciliation 226

1. Abstract

This project aimed to investigate the use of plant growth regulators to manipulate the timing of berry harvest to alleviate climate change-associated compressed harvest seasons with, benefits for winery intake scheduling and fruit quality. Trials in commercial vineyards were used to optimise spray treatments that significantly delayed veraison/ripening without negative effects on berry and wine composition and sensory character. These data will also be used to assist product registration for commercial use. Non-volatile developmental stage-specific berry metabolites were identified that could be used to develop an in-vineyard test to define the optimal timing of ripening delaying treatments.

2. Executive summary

The main aim of this project was to find a reliable method to delay grape harvest in a selective manner to combat some of the deleterious effects of climate change. A particular target was the hastening of ripening/harvest which has led to harvest season compression and associated problems with berry and wine quality. We know from previous work that some auxinic compounds can delay the onset of ripening and therefore harvest. Here we describe work conducted to develop the use of the auxin, 1-naphthalene acetic acid (NAA) as a commercially viable tool to delay ripening in a targeted manner. To broaden the scope, we tested two reagents liable to advance ripening/harvest and two to delay it. The other reagent that showed promise as a delayer of ripening, Ethrel, was shown to delay ripening but timing the spray treatment to be effective was particularly difficult and whole vine sprays showed that it hastened leaf senescence. NAA was the most effective in delaying harvest and did not damage leaves. A single, well-timed spraying of the fruiting zone is all that is required, reducing spray volume and reagent input.

Apart from delaying harvest, with the benefit of stretching the harvest season and alleviating winery intakes pressures, NAA-treatment has other positive benefits. NAA-treated berries tend to be larger at harvest as they undergo less shrinkage and appear more structurally sound. The rachis of NAA-treated bunches stays green and apparently healthier much longer than that of the untreated bunches. Although not formally proven, this could have an influence in mitigating bunch stem necrosis. Our experiments showed that the delaying effect on ripening/harvest may be through changes in gene expression and enzyme activity of cell wall modifying enzymes. These changes indicate that cellulose synthesis, required for the primary cell wall, was increased by NAA while the activity of enzymes that modify and degrade pectin, which is important for tissue structure, was reduced. The increased rigidity of the walls may contribute to the delaying of ripening by slowing berry expansion.

NAA-induced harvest delay causes only modest changes in berry composition and wine flavour/aroma. We showed through full and partial peduncle girdling experiments that slowing or halting berry sugar accumulation does not, in many cases, greatly alter berry composition and wine character. The observed increase in rotundone levels in NAA ripening-delayed Shiraz fruit may be a direct effect of NAA on sesquiterpene synthesis, leading to increased peppery notes in the wine. Some of the changes in wine composition may be due to longer hang times e.g. the slightly lower IBMP concentration in NAA harvest delayed wine that may be a result of increased hang time. Lower levels of IBMP, which imparts an undesirable green character to Cabernet Sauvignon wines, would be seen as a positive outcome. Another interesting effect of the NAA harvest delaying treatment, possibly related to changes in berry cell walls, is that the treatment tends to increase Brix synchronicity (i.e. the individual berries tend to have more similar Brix compared to the untreated) and to decrease berry weight synchronicity. As the development of flavour/aroma compounds tends to be linked to sugar concentration, this might be an advantage in terms of wine flavour/aroma with fewer 'too green' and 'too ripe' flavours.

Apart from these effects on berries and wine, we spent considerable effort attending to the practical issues surrounding NAA use in industry. We have shown that the timing of the application is important to the treatment efficacy, with spraying as close as possible to veraison resulting in the longest delay in harvest. However, if the ripening in the vineyard is very asynchronous, this has to be taken into account, as once a berry commences ripening, NAA treatment cannot delay its harvest. Spraying too late in this situation may make the ripening even less synchronous. Timing of application is very important and in a changing climate, predicting veraison can be difficult. To this end we have performed an extensive analysis on the non-volatile berry metabolites that change in concentration during the late pre-veraison

stage. A number of compounds were discovered that increase or decrease in concentration during this period. These markers of developmental stage could be developed into a test for the timing of the just pre-veraison spray application.

The compatibility of NAA with a selection of fungicides, an insecticide and different wetting agents was tested by combining each of these with NAA in a spray treatment, to assess its performance. There was no decrease in the effectiveness of NAA due to the presence of any of the fungicides or the insecticide and no particular wetting agent was markedly more efficacious, but this could be tested further on a larger scale trial.

It would be expected that the length of delay in harvest would be affected not only by the timing of application, but also by the concentration of NAA applied. Our initial studies were completed using 100 ppm NAA, however, we subsequently showed that this can be reduced to 50 ppm and still produce a significant and practically useful delay in harvest timing. The dose response curve shows that the delaying effect of NAA on ripening does not increase linearly at higher dosage levels. Reducing the amount of NAA applied reduces the quantity and cost of reagent applied and the concentration of any residues in the berries and wine. NAA is used to control various aspects of fruit development in apples and pears and is considered to be a soft/safe option. However, residues are always an issue in registering an agent for use in a different crop. Reducing the amount applied is therefore an important consideration. We have shown that the residue levels in grapes are below the level of detection and are low in wines. We have shown that the residues are not located on the berry surface. Studies of NAA residue in bottled wine have shown that it decreases quite rapidly with time. A registration pre-application advice has been commissioned from the APVMA, residues have been tested by an accredited laboratory, and the effort to progress this is ongoing.

In this project we have done extensive field and laboratory studies to develop a cheap, adaptable and reliable method to delay berry ripening and hence harvest time. Apart from expanding the harvest window and relieving pressure on winery intake caused by compressed harvest seasons, and improving berry and wine quality through harvesting at the optimal time, there are a number of other potential economic benefits, as listed above.

There are other ways to tackle some of the agronomic issues faced by the grape and wine industries. Classical breeding is not currently popular, but genetic methods offer powerful tools to discover target genes for the new technique of gene editing. Targets for editing that would address events such as the timing of budburst, flowering, veraison, the rate of sugar accumulation during the ripening phase etc. will be immensely valuable in the near future and could be used to manipulate not just phenological stages but other parameters as described in this report. Identifying the gene targets for editing the desired traits is an essential part of the process. As a first step we phenotyped a wide range of characters in a breeding population over two years and have demonstrated that there is sufficient natural variation to make large adjustments to traits such as harvest timing. The phenotypic data can be used in future work to define the key genes in these processes and the precise changes in the genes that lead to the desired trait, for example, late budburst leading to a later harvest.

We would like to acknowledge the generous support of our scientific and industry collaborators. We thank Treasury Wine Estates and Chalk Hill Wines for access to their vineyards for our studies; Associate Professor Matthew Tucker, University of Adelaide (who acknowledges support from the ARC Centre of Excellence in Plant Cell Walls (CE1101007)) and his staff for their studies involving the changes in grape cell walls due to NAA treatment; Associate Professor Sue Bastian, University of Adelaide, and staff for

wine sensory analysis. Professor Mario Pezzotti's research group, University of Verona, Italy very kindly conducted genome-wide gene expression analysis of NAA-treated fruit for us. This work benefited from the networking activities within the European-funded COST ACTION FA17111 'Data integration to maximise the power of omics for grapevine improvement'. CD acknowledges the support from the Veneto Region project 1695-11-11-2018. We acknowledge Maia Rabinovich for assistance with the *ex planta* experiment, Giacomo Betti, CSIRO for conducting canopy coverage measurements by drone and Emily Nicholson, CSIRO for assistance with wine volatile analysis.

3. Background

The use of fossil fuels has led to increased atmospheric carbon dioxide levels and increased temperatures, both of which can affect plant growth and metabolism. Grapevines are sensitive to temperature variations and as a result, striking changes in grapevine phenology and composition have been observed, as have consequent changes in wine composition and sensory properties (Mira de Orduña 2010; Venios *et al.,* 2020). Increased temperatures appear to be the main factor causing changes to grapevine phenology (Jones *et al.,* 2005; Petrie and Sadras 2008; Cook and Wolkovich 2016). The importance of increased temperatures on berry and wine composition will only increase as warming continues (Duchêne and Schneider 2005; Mira de Orduña 2010; Ausseil *et al.,* 2021). Although there may be some benefits from increased temperature on the sensory properties of wines from fruit grown in what where once very cool regions, much of the world's wine production is grown under already warm climates and so measures are especially required to alleviate climate change issues in these regions.

Apart from changes to wine sensory properties, increased temperatures have also resulted in earlier harvest times mainly due to earlier budburst, flowering and veraison, but there is also evidence of more rapid ripening (Cameron *et al.,* 2020; Petrie and Sadras 2008, 2016). This has shifted ripening into warmer parts of the growing season further increasing the problems caused by increased yearly temperatures. Higher temperatures have resulted in berries with increased sugar levels but with lower flavour potential. In an attempt to improve flavour, fruit has been allowed to remain longer on the vines. However, longer 'hang times' have resulted in wines with increased alcohol concentration, which is seen as a potential health issue and can cause some wines to be perceived as 'hot' on the palate. In addition, high sugar levels can result in problems with increased ethanol concentration during fermentation. The changes in sensory properties can result in fruit and wine of reduced commercial value.

In addition to potential quality issues, the phenomenon of harvest season compression has caused logistical problems for grape growers and winemakers. Cultivars of wine grapes (*Vitis vinifera* L.) that previously had ripened later in the season seem to be more affected by increased temperature than earlier ripening cultivars and as a consequence, many cultivars are now ripening at much the same time rather than over a spread-out harvest season (Petrie and Sadras 2016; Cameron *et al.,* 2020). This compression causes problems for harvesting and winemaking capacity as the peak load period is shorter in duration, which places additional pressure on harvesting and winemaking resources. It can also potentially reduce the value of the product (see below) and can have a negative financial impact through increasing costs.

The equation Genotype + Environment + Management = Phenotype (GEM=P) describes the three influences on the yield and composition of grapes. We have seen above how the environment is changing and how this is having large effects on berry ripening. A range of viticultural management practices including, shoot trimming, late pruning, shading and the use of anti-transpirants have been developed to try to delay harvest times, but with mixed success (Gu *et al.,* 2012; Frioni *et al.,* 2016; Pineau *et al.,* 2017; Moran *et al.,* 2017; 2018; Palliotto *et al.,* 2017; Petrie *et al.,* 2017). Many of these practices are labour intensive and can be expensive, are only partially effective under some conditions, can be difficult to time and may result in reduced yield in subsequent seasons (Frioni *et al.,* 2016, 2019; Gatti *et al.,* 201; Palliotto *et al.,* 2017; Petrie *et al.,* 2017). Other methods to relieve climate change-induced seasonal harvest compression include harvesting either earlier, or later than desirable, followed by blending to reduce green or overripe characters and reduce alcohol concentration. Recent regulatory changes have allowed Australian winemakers to add limited amounts of water to ferments under certain conditions to reduce

sugar (and hence alcohol) concentration and potential problems with stuck ferments. There are also post-fermentation techniques that allow removal of ethanol but there is the risk of the removal of flavour compounds during this process.

As a consequence of the problems arising from climate change, the grape and wine industries need additional solutions to a range of issues that threaten their sustainability and profitability. Auxins are plant growth regulators (PGRs) involved in a wide range of developmental processes, including fruit development, and responses to various stimuli. Auxins are usually associated with growth during the early stages of fruit development but also have other roles (Pattison *et al.,* 2014). For example, auxins are involved in the growth of roots and aerial parts of vines and in the control of grape berry ripening which has been the focus of numerous recent studies. It has been demonstrated that indole-3-acetic acid (IAA) concentrations are high in flowers and young berries but decrease rapidly to be at low concentrations prior to veraison (the initiation of ripening) (Böttcher *et al.,* 2010). Auxins have been hypothesised to be inhibitors of berry ripening initiation as the decrease in IAA concentration before veraison appears to be a prerequisite for ripening to commence (Böttcher and Davies 2012b). In berries, it has been demonstrated that IAA can be inactivated by conjugation with amino acids (Böttcher *et al.,* 2010) and broken down by oxidation. Supplementation of the endogenous auxin through spray application is an option to extend the pre-veraison period and therefore delay ripening and harvest. Treatment with auxins, for example through the application of the synthetic auxins 1-naphthaleneacetic acid (NAA) and benzothiazole-2-oxyacetic acid (BTOA) during the pre-veraison period delayed the onset of grape berry ripening and hence delayed harvest (Böttcher *et al.,* 2011a; Böttcher *et al.,* 2011b; Coombe and Hale 1973; Davies *et al.,* 2015).

Attempts to use the endogenous auxin IAA (which has the advantage of already being present within vines) to delay ripening/harvest have so far been unsuccessful. The most likely reason seems to be that the endogenous IAA is quickly inactivated by the mechanism discussed above. This is not surprising as plant hormones are usually tightly controlled because they are active at low concentrations and are therefore under homeostatic control within fairly narrow limits that depend on the tissue/organ/stage of development.

Given that endogenous IAA is biologically labile, it was proposed that control of ripening in a commercial setting might be possible using a synthetic analogue of IAA that is not so rapidly inactivated within the berry. NAA is an auxin used in horticulture e.g. in the apple and pear industries for fruit set, fruit thinning and fruit retention near to harvest (depending on the timing of application) and is considered to be safe and reliable.

In brief, the concept for the use of NAA to control ripening involves a spray application of NAA at some time before veraison, to delay ripening by a few weeks. This process could be used to spread the load on harvesting and winemaking capacity and could provide substantial economic benefits.

Although we have previously demonstrated that NAA has the potential to be an effective agent in controlling ripening and therefore harvest, considerable testing and refinement is required to demonstrate treatment efficacy and reliability, to better understand the mechanism of NAA action and metabolism in the berry, to refine conditions of use, to test for any residues and to define any effects on vine health, berry and wine composition and on wine sensory characters.

We have shown that the timing of the application of NAA is important to the efficacy of the treatment in delaying ripening. This is further explored in this project in some detail. We know that the timing of

spraying in relation to the onset of ripening (veraison) is likely to be the key factor. Predicting veraison date is not always straightforward as seasonal differences mean that the date of veraison for any season can vary in either direction from the historical average date. We have done extensive research to find biochemical markers that predict the initiation of ripening. The aim is to identify molecules that change significantly in concentration (either increasing or decreasing) shortly before veraison and as such can be used as indicators of ripening date. The outcome would be that the identification of reliable markers predicting veraison could be the basis of a simple test that could be used by growers and winemakers to accurately predict veraison. Apart from allowing time for managers to conduct the NAA treatments at the ideal time, this test may also be useful in planning harvesting and processing of all fruit.

The last component in the GEM=P equation is the genotype of the vines. The genotype has the potential to be more influential in controlling the effects of global warming than management techniques. In the currently available germplasm, there is additional untapped genetic diversity that could be used to tackle the issues that currently impact the grape and wine industries. However, many of these traits reside in cultivars that are not considered as elite and do not produce wine that is preferred by consumers. So, in addition to the approach of using NAA as a means of delaying veraison, and therefore harvest, we have begun to investigate the possibility of developing elite cultivars with different, genetically determined harvest times. As a precursor to this we needed to measure how much diversity there was in the available crosses for the range of traits we deemed potentially useful. These traits include the timing and length of budburst and flowering periods, the timing of veraison and 20 ^{o}Brix, the time from flowering to veraison, the rate of sugar accumulation, berry weight, berry weight gain/loss after reaching 20 ^{o}Brix, synchronicity of Brix, canopy size and yield per canopy size. Shifting the harvest period by having cultivars with delayed budburst/flowering would have the additional benefit of making frost damage and reduced fruit set less likely. Delaying veraison would delay harvest as would happen if cultivars had a slower rate of sugar accumulation, and the latter approach may also improve fruit/wine quality. Ripening synchronicity and yield are also traits that could be further studied. These data will be used for mapping studies where the first step will be to identify the region of the genome most likely to have a genetic variation that explains the observed phenotype. The initial focus will be on identifying the region of the genome that is responsible for the desired trait e.g. delayed budburst and then identifying the key gene and its function. The ultimate goal is to use this knowledge to modify existing premium cultivars via gene editing, to produce cultivars with the same name but improved characteristics.

4. Project aims

The overarching objective of this project is to develop optimised formulations and application methodologies to provide tools for grapegrowers that can be used to expand the harvest period, prevent ripening from occurring during the hottest part of summer and produce fruit of enhanced quality. This will be achieved by:

1. Fine-tuning ripening-delaying treatments, developed during our current and previous GWRDC- and Wine Australia-funded projects, using NAA and other PGRs (including any new potentially useful PGRs) and adapting treatments to a commercial setting. This includes preparing the background data (e.g. residue levels, application recommendations) for registration and coordinating the registration process for the commercial use of NAA and/or other ripening-delaying formulations.

2. Building on promising outcomes from our previous studies, further investigating the effect of ripening-delaying treatments on fruit quality parameters, focussing on a selection of flavour/aroma compounds and the improvement of ripening synchronicity. The knowledge obtained will be used to optimise treatment regimes to enable the management of grape composition as it relates to wine style.

3. Developing mass spectrometry-based methods for the analysis of non-volatile grape metabolites. This capability will be used to develop metabolite markers to allow the classification of pre-veraison grapes into specific developmental stages, identify those stages most responsive to PGR treatments and lead to the methods which will allow vineyard managers to reliably predict the best time to treat the fruit for optimal effect on ripening. Any future in-vineyard tool developed to assist in accurate timing of treatments would be suitable for commercialisation.

4. The original aims were tuned during the project to include testing the use of PGRs and other compounds to delay the onset of budburst as an additional means of delaying harvest and the phenotyping of varietal and breeding collections to investigate the genetic variation available in key vine and berry developmental steps (e.g. the timing of budburst) to enable a genetic approach (e.g. gene editing) to altering the timing of ripening/harvest in commercial cultivars.

Output	Activity
2017/18	
A) Information on samples from final year of CSP 1401	Complete any work unfinished from previous project (CSP 1401) that feeds into the new project including analysis of wine made in final year, such as, the GC-MS evaluation of flavour/aroma compounds and any wine sensory analysis to quantify any differences caused by the delaying treatments.
B) Field experiment to test important treatment parameters	Conduct field-based experiments using Shiraz to test the effects of different NAA concentrations and commercial NAA formulations. Analyse Berry Brix, weight, acids, colour, pH to measure the effects on berry ripening.
C) Wine made from harvest samples of treated and Control fruit and data analysis of flavour/aroma compounds	Make small-scale wines from fruit produced in above trial, all three replicates of treatment and Control samples. Measure differences in flavour compounds (e.g. rotundone) as required using GC-MS analysis; some of this work will continue into Year 2. These analyses will determine if the delaying treatments have resulted in measurable differences in volatile compounds involved in wine sensory properties.
D) Data on residues of applied NAA in both fruit and wine of treated sample	Extract and measure residues of the applied PGR, both free NAA and its major amino acid conjugate NAA-Asp, in both grapes and wine using LC-MS techniques. This analysis will continue into Year 2.

E) Approval from regulating authority for larger trials	Negotiate, as required depending on outcome of 2016/17 trials, with regulating authority for approval for larger trials in order to do field experiments in a situation more closely resembling the commercial setting. Engage CSIRO B&D support to aid this process; design trials, identify suitable sites. Engage grape/wine and agrochemical industries in this process as required.
F) Data from first round experiment to identify metabolite markers of berry developmental stage	Identify candidate non-volatile metabolite markers in Shiraz, using LC-MS-QTOF, that indicate the stage of berry development for optimal timing for NAA application in the field.
2018/19	
A) Knowledge of the effects of different concentrations of NAA on sensory, biochemical properties and berry and wine volatile composition	Conduct analyses of samples from previous season. This will include measuring differences in biochemical properties, including flavour/aroma compounds as required (GC-MS), the analysis of NAA residues in grapes and wine (LC-MS), analysis of samples for treatment time marker metabolites (LC-MS-QTOF). After statistical analysis use the previous year's data in planning of the next season's experiments. Involve industry in wine sensory analysis.
B) Data and samples from larger scale experiment testing delaying treatment in Cabernet Sauvignon in field	Conduct larger scale field trials in a commercial setting using parameters: NAA concentration, spray timing etc. based on previous year's results. Parameters to test could include different timing of single applications (including measurement of ripening synchronicity) and/or the treatment of whole vines for multiple years.
C) Knowledge of the effects of delaying treatments on berry and wine parameters	Sample the trials throughout the season and measure berry development parameters. Make wine from the trials and commence analysis of flavour/aroma compounds by weight spectroscopy as required. Progress is subject to date of wine delivery.
D) Second round of experiments to identify metabolite markers commenced	Continue LC-MS-QTOF analyses to identify suitable metabolite markers of best timing for NAA application. Purify and identify unknown candidate markers by NMR. Collect more berries from Shiraz and Cabernet Sauvignon to broaden search and test if any markers identified in Shiraz are suitable for another cultivar.
E) Knowledge of most effective spray adjuvant to be used in NAA spray treatments	Conduct a larger scale field trial to test the most promising spray adjuvants to be used with NAA application. Sample berries during development and measure/analyse berry developmental parameters.
F) Plan of action developed with Company A regarding NAA use in wine grapes	Undertake discussions with Company A for the registration of NAA for use in wine grapes.
2019/20	
A) Revised plan for the processes required for NAA registration for use by the APVMA	Revise and further develop plan for the registration of NAA use to delay berry ripening in Australia following advice on registration requirements from the APVMA. This will be done through discussions between WA, CSIRO, the potential commercial partner Company A, a specialist chemical registration consultant and the APVMA recognised contractor for residue testing.
B) Experimental data on the effects of NAA on grape berry ripening to support APVMA registration approval	Conduct field experiments in accordance with the outcomes of a) which are required for and essential to progressing the registration for the commercial use of NAA to delay berry ripening and therefore reduce the economic impact of climate-induced changes in grape berry development.
C) Knowledge of the changes with time of the concentration of NAA residues in bottled wine, the effects of NAA treatment on sesquiterpene production in	Ongoing monitoring of NAA residues in wine to determine decay with time.

ripening delayed fruit and the relationship between IAA levels and ripening timing in different varieties	Measure IAA and its metabolites in cultivars with different ripening times (different vineyards) to determine if auxin concentration is the sole determinant of ripening timing. Conduct experiments to determine the effect of ripening delay on sesquiterpene synthesis.
D) Knowledge of the factors determining budburst and its effect on harvest timing	Conduct a literature search regarding the control of bud dormancy/budburst with the idea of trying to delay it (prevent frost damage, delay harvest etc.). Conduct experiments investigating the role of PGRs in budburst and the potential to regulate budburst in a controlled manner by PGRs and other means.
E) First metabolite markers for optimizing NAA spray application timing identified and robustness assessment commenced	Continue LC-MS-QTOF analyses, HPLC purification and compound identification via NMR from Shiraz grape extracts to identify suitable metabolite markers for optimised NAA application timing. Test marker robustness across seasons and cultivars using Shiraz and Cabernet Sauvignon berry samples. Subject to the results obtained from these marker studies modify extraction and purification protocols to extend marker search to more polar metabolites.
2020/21	
A) Field experimental data testing the effectiveness of lower NAA concentrations (to decrease residues) on delaying grape harvest and data on Ethrel effectiveness, for same	Conduct field experiments which are required for progressing the use of PGRs to delay berry ripening and therefore reduce the economic impact of climate-induced changes in grape berry development. Low range NAA treatments in field, analyse effect on ripening delay and residue levels. Further testing of Ethrel to delay ripening, analyse residues (previous results show that it can delay ripening). Ethrel may not be as effective as NAA but there are MRLs for wine for all major wine export markets.
B) Knowledge of the changes with time of the concentration of NAA residues in grapes and bottled wine using GLP assay	Ongoing monitoring of NAA residues in wine to determine decay with time. Testing of grape and wine samples for NAA residues with a GLP assay will be used to inform the next steps in registration/commercialisation as the question of residues is crucial. Use this knowledge to continue to promote the potential of NAA in Australia and overseas through collaborations.
C) Potential of various reagents to delay budburst determined. Knowledge of genetic variability of budburst timing and factors	Conduct experiments to further test the effects of PGRs and other reagents in delaying budburst. Use phenotypic data and genetic analysis to define variation in budburst timing in germplasm, identify QTLs associated with the phenotype and identify candidate genes underlying the QTLs.
D) Two or more metabolite markers for optimizing NAA spray application timing identified and robustness assessment completed	Assess marker identified in previous year in berry samples from an additional 2 seasons and explore suitability for use in 'spray-timing assay'. Use column chromatography, HPLC purification and LC-MS-QTOF analyses modified for polar compounds to identify further suitable metabolite markers for optimised NAA application timing. Test marker robustness across seasons and cultivars using Shiraz and Cabernet Sauvignon berry samples.
E) Phenotyping and genetic mapping of basis of traits that control harvest timing	Collect data on key developmental processes that might affect harvest timing using appropriate germplasm Sample leaf material from germplasm for DNA extraction and genotype-by-sequencing analysis. Initiate the mapping of genome regions that control traits important for the timing of harvest.
2021/2022	

A) Field experimental data testing the use of NAA in delaying ripening/harvest	Conduct field experiments which are required for progressing the use of NAA to delay berry ripening and therefore reduce the economic impact of climate-induced changes in grape berry development.
B) Knowledge of the changes with time of the concentration of NAA residues in grapes and bottled wine using GLP assay	Ongoing monitoring of NAA residues in wine to determine decay with time (the results of GLP testing have confirmed that our in-house isotope dilution method is accurate and reliable). Testing of grape and wine samples for NAA residues from the previous season's studies in Shiraz and Cabernet Sauvignon. Use this knowledge to continue to promote the potential of NAA in Australia and overseas through collaborations and in future proposed discussions flagged by the Company B.
C) Potential of new candidate reagents to delay budburst determined. Knowledge of genetic variability of budburst timing and factors	Conduct in-field experiments to test the effects of other reagents with the potential to delay budburst. Continue analysis of candidate genes identified by GWAS for budburst timing.
D) Identity of current marker compounds confirmed. New, positive marker for optimal spay timing identified	Column chromatography to further purify a candidate positive marker for optimal timing of NAA treatment to delay ripening is underway. After partial purification and concentration this compound will have to be of sufficient purity and quality to allow precise identification by nuclear magnetic resonance spectroscopy.
E) Phenotypic data from two years of field studies analysed. Best targets for vine improvement identified. Genetic mapping of basis of traits that control harvest timing	Analyse two years of data on key developmental processes that might affect the timing of ripening and harvest, identify the extent of variation in breeding population. Extend the mapping of genomic regions that control traits important for berry development and the timing of ripening and harvest (with CSA 1701-1.2) and conduct studies for marker development. Provide complete set (131) of wines from breeding population for two consecutive years for volatile analysis by CSA 1701-1.2. Test the use of CRISPR-Cas system to modify an auxin pathway gene potentially involved in the control of ripening (see CSA 1701-1.7).
F) Submission of final project report. Communication of results as applicable	Prepare final report Publish at least two scientific papers Disseminate results contained in final project report to industry where possible (e.g. AWRI Roadshow, industry seminars)

5. Method

The methods presented here are common to more than one of the experiments detailed below. Additional Materials and Methods specific for each experiment or trial (e.g. field plans, specifics of individual experiments) are given at the start of each section.

5.1 Spray treatments

All reagents were sprayed using hand-held devices or a backpack sprayer. The reagents applied are specified for each experiment but included a wetting agent. Bunches were sprayed to runoff, for whole vines, and all leaves and bunches were treated. A replicated, random layout was used and treated vines or bunches were designated with different coloured and patterned flagging tape.

5.2 Basic berry measurements

Total soluble solids (TSS) measurements (measured as ^{o}Brix) were made using an RFM710 digital refractometer (Bellingham Stanley, Turnbridge Wells, UK). Berries were weighed using a precision digital balance (PB3002-S; Mettler-Toledo, Melbourne, Australia). Frozen berries were ground to a powder using an IKA A11 basic analytical mill (IKA, Staufen, Germany) and assayed for anthocyanin content as described by Davies *et al.* (2015). pH was measured by electrode, glucose, fructose, primary assimilable nitrogen and total yeast assimilable nitrogen (total YAN) were determined using K-FRUGL, K-PANOPA and K-LARGE kits (Megazyme International Ireland Ltd., Wicklow, Ireland) as described by Boss *et al.* (2014). An OenoFoss instrument (Foss, Hillerød, Denmark) was also used to measure basic berry parameters as per the manufacturer's instructions.

5.3 Analysis of plant macro- and micronutrients

The concentration of plant macro- (Ca, K, Mg, Na, P, S) and micronutrients (B, Cu, Fe, Zn) was determined by nitric acid digestion and inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis of 2 g of thawed, powdered grape tissue as described by Walker *et al.* (2019), but the instrument used was an Agilent 5100 ICP-OES.

5.4 Amino acid analysis

Amino acids from powdered berry tissue (200 mg) were quantified as described by Boss *et al.* (2015). Since proline could not be reliably detected by this method at low concentration, this amino acid was measured according to the ninhydrin method of Rienth *et al.* (2014) with the following modifications: 500 µL of diluted juice, formic acid and ninhydrin solutions were used for the reaction, and absorbance was measured at 520 nm using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany).

5.5 Small-scale winemaking

Small-scale winemaking of the harvested Shiraz and Cabernet Sauvignon berries and the analysis of basic wine parameters was conducted by WIC Winemaking Services (Urrbrae, SA, Australia) using the following protocol. Harvested fruit was cooled overnight at 4°C. SO_2 levels were adjusted to approximately 50 ppm during destemming/crushing. Small amounts of tartaric acid were added to the Control and G2 musts to adjust the pH to approximately 3.6 and titratable acidity (TA) to approximately 6 g/L. Yeast strain EC1118 (Lallemand, Edwardstown, SA, Australia) was added to a concentration of 200 ppm. The ferment was conducted on skins at 18-20°C, the cap was plunged approximately 20 times per day. The must was pressed at 2 °Baume, fermented to dryness and racked. SO_2 was added to 60 ppm and the wine stabilised at 0°C. The wine was then racked, SO_2 adjusted to 80 ppm, filtered and bottled with Stelvin closures (Amcor, Hawthorn, VIC, Australia). When required, a malolactic fermentation was also conducted.

5.6 Laboratory-scale winemaking and headspace volatile analysis

Small-scale (25 g ground berry material) fermentations were carried out as described by Boss *et al.* (2014) except that the glucose and fructose concentrations were normalised so that all fermentations had the same final sugar concentration. Glucose, fructose, primary assimilable nitrogen and total yeast assimilable nitrogen (total YAN) were determined using kits as described by Boss *et al.* (2014). Ethanol, total acidity, malic acid, glucose/fructose, volatile acidity and pH were measured in the finished wines using an OenoFoss instrument (Foss, Hillerød, Denmark) following the manufacturer's instructions.

5.7 Quantification of methoxypyrazines and rotundone in grape tissue

Frozen, powdered Cabernet Sauvignon grape berry tissue was used for the quantification of the predominant grape methoxypyrazine, 3-isobutyl-2-methoxypyrazine (IBMP), as described by Armstrong *et al.* (2021) and the equivalent Shiraz samples were used to quantify rotundone following the method of Davies *et al.* (2015).

5.8 Analysis of headspace volatiles

Headspace volatiles of the wines were analysed by solid-phase microextraction (SPME) GC/MS as described by Boss *et al.* (2014).

5.9 RNA extraction, cDNA synthesis and qRT-PCR

RNA extraction, cDNA synthesis and qRT-PCR were performed as described previously (Böttcher *et al.,* 2013b) with modifications as described by Böttcher *et al.* (2015b).

5.10 Quantification of NAA in grapes

100 mg of tissue was extracted with 1 mL of 60% (v/v) iso-propanol containing 500 pmol of 50 µM $^{13}C_2$-NAA-Asp standard for 2 h at 4°C with mixing. After centrifugation, the supernatant was removed and the pellet re-extracted with 1 mL of 60% (v/v) iso-propanol for 1 hr at 4°C, following centrifugation the supernatants were combined and most of the ethanol evaporated before acidification with 50% HCl which was then extracted three times with ethyl acetate. The pooled ethyl acetate fractions were dried before resuspension in 100 µL of MeOH/water/acetic acid (60:39:1, v/v/v) and assayed by LC-ESI-MS/MS (Böttcher *et al.,* 2012c).

5.11 Quantification of NAA in wine

To 2 mL of wine 100 µL of 10% HCl was added, mixed (final pH below 2) and to 500 µL of acidified wine 500 pmol of $^{13}C_2$-NAA-Asp standard was added. This solution was then extracted three times with ethyl acetate. The pooled ethyl acetate fractions were dried before resuspension in 100 µL of MeOH/water/acetic acid (60:39:1, v/v/v) and assayed by LC-ESI-MS/MS (Böttcher *et al.,* 2012c).

5.12 Data analysis

Any possible effects of NAA treatment on grape and wine parameters, wine volatiles and gene expression were tested for statistical significance by ANOVA, followed by Duncan's or Tukey's post hoc test (IBM SPSS software ver. 20; IBM Australia, Sydney, NSW, Australia) or Student's t-test (Microsoft Excel, ver. 2008; Washington, USA).

6. Results and discussion

As there are diverse trials and experiments in this document, a small specific introduction is included to provide context for the reader.

6.1 The testing of multiple PGRs to alter veraison/harvest date in Cabernet Sauvignon and Shiraz

6.1.1 Introduction

The reasons for wanting a cheap and reliable means for delaying veraison and therefore grape harvest were outlined in the Background (Section 3). Although we have shown that NAA can effectively delay harvest when applied at a suitable, pre-veraison stage (Böttcher *et al.,* 2011a, b, 2012a; Davies *et al.,* 2015) it was deemed sensible to investigate additional PGRs that might also be able to delay harvest. Having more than one avenue to delay harvest could be beneficial in the event that NAA causes problems with vine health/growth, in some way negatively affects berry or wine quality or if other reagents might be either more efficient or, for a range of reasons may be easier to commercialise for use.

In addition to the already documented use of NAA as a 'harvest delayer' ripening can also be delayed by applying the ethylene-releasing reagent Ethrel ((2-chloroethyl)phosphonic acid) at a particular time before veraison (Hale *et al.,* 1970, Böttcher *et al.,* 2013b). Ethrel has been used in 'climacteric' fruit to alter fruit development e.g. to hasten ripening and improve skin colouration, to loosen fruit to facilitate harvesting. However, grapes are non-climacteric fruit, and the delaying of ripening in response to ethylene appears to work indirectly, with the released ethylene increasing the concentration of the endogenous auxin, IAA, through an increase in the activity of the auxin biosynthesis pathway, thus delaying ripening in a manner similar to NAA, albeit indirectly. The timing of this application is more difficult to judge than for NAA as the window for success seems to be narrower. However, if the timing of application that leads to a useful delay in harvest could be better defined it could supplement or offer an alternative to NAA as a ripening delaying agent. Why endogenously synthesised IAA delays ripening but sprayed IAA does not, is not known but may relate to the ethylene-induced increase in IAA biosynthesis continuing for a longer period, whereas the applied IAA is metabolised during a relatively short window after the application.

Other agents can be used to advance ripening, which is another way of extending the harvest season and relieving the pressures on winery capacity during peak periods. Aminoethoxyvinylglycine (AVG) is a naturally occurring PGR that reduces ethylene production by inhibiting an enzyme crucial to its synthesis in the berry and at the same time also reduces the synthesis of IAA by inhibiting a functionally related enzyme in the auxin biosynthesis pathway. If applied at the correct time, AVG can advance ripening (Böttcher *et al.,* 2013b). Similarly, another naturally occurring PGR, abscisic acid (ABA), can also advance ripening when applied pre-veraison (Wheeler *et al.,* 2009). In all cases the concentration and particularly the timing of application are the keys to success.

These experiments were designed to test the effects on ripening and harvest timing of the PGRs outlined above and to assess any effects of treatment on berry composition using Cabernet Sauvignon and Shiraz vines. The aims were to:

- Test the effect of NAA in delaying veraison/harvest
- Test the application of the ethylene releasing compound Ethrel as a possible reagent for delaying veraison/harvest

- Test the application of AVG as a means of hastening veraison/harvest with the potential to also improve skin colour development
- Test the effect of ABA as an agent to hasten ripening/harvest

6.1.2 Materials and methods

Cabernet Sauvignon and Shiraz in nearby blocks in the Barossa Valley were used for this trial. Treatments were conducted using a randomised triplicate trial design across three rows (one replicate of each treatment in each row). One panel was used per replicate.

The treatments were as follows:

Control (wetting agent (**WA**) only) Vitiwet (SST Products, South Melbourne, Australia)

NAA + WA (single pre-veraison treatment time) NAA 50 mg/L (1-naphthaleneacetic acid as NAA20, Grochem, Port Melbourne, Victoria, Australia)

AVG + WA (two pre-veraison treatment times) AVG (aminoethoxyglycine) as Retain® 1.67 g/L (Valent BioSciences, Libertyville, IL, USA)

Ethrel + WA (two pre-veraison treatment times) Ethrel® (2-chloroethylphosphonic acid) (Bayer Crop Science, East Hawthorn, Victoria, Australia) 300 µL/L

ABA + WA (one pre-veraison treatment time) ABA (abscisic acid) 400 mg/L (A. G. Scientific (San Diego, CA, USA)

ABA and NAA were first dissolved in 0.5 mL ethanol prior to dilution in the water and wetting agent solution.

Two separate treatment times were initially proposed for Ethrel and AVG, as they are the two compounds for which we were the least confident regarding the timing of treatments for best effect (Figures 1, 2, 3). These two compounds are less long-lasting than NAA and so defining the most effective treatment window can be more problematic. In addition, we had not worked with the vines used in this study before and so it was more challenging to accurately predict ripening time than in vineyards more familiar to us. Given that the timing of application is so critical to the effects of these treatments on berry development/ripening this was an important factor. As a result of veraison occurring significantly earlier than we predicted, the Ethrel 2 treatment was not completed.

Row 1	Row2	Row3
AVG 1	AVG 2	Control
Ethrel 2*	Ethrel 1	AVG 1
NAA	Control	Ethrel 2*
AVG 2	ABA	Ethrel 1
ABA	NAA	AVG 2
Control	AVG 1	NAA
Ethrel 1	Ethrel 2*	ABA

Figure 1. Triplicated, randomised trial design for both vineyards.

*Note that the Ethrel 2 treatment was not applied due to the stage of development being too late for effectiveness.

Treatment	Details	Spray	Spray	Spray
		21 Dec	28 Dec	4 Jan
Control	0.01% Vitiwet (WA)			
				1
NAA	50 mg/L + WA			
				1
ABA	400 mg/L + WA			
			1	2
AVG 1	125 mg/L + WA			
		1	2	3
AVG 2	125 mg/L + WA			
			1	2
Ethrel 1	288 mg/L + WA			
				1

Figure 2. Spray chart showing treatment times for Cabernet Sauvignon. The concentrations of the various PGRs are given as mg/L active ingredient. The shaded boxes indicate the dates of spaying events for each of the treatments, the numerals indicate the number of sprays applied e.g. Control, NAA and Ethrel were only treated once. WA = wetting agent.

Treatment	Details	Spray	Spray	Spray	Spray
		21 Dec	28 Dec	4 Jan	11 Jan
Control	0.01%				
	Vitiwet				1
	(WA)				
NAA	50 mg/L				
	+				1
	WA				
ABA	400 mg/L				
	+		2	1	
	WA				
AVG 1	125 mg/L				
	+	3	2	1	
	WA				
AVG 2	125 mg/L				
	+		2	1	
	WA				
Ethrel 1	288 mg/L				
	+			1	
	WA				

Figure 3. Spray chart showing treatment times for Shiraz. The concentrations of the various PGRs are given as mg/L active ingredient. The shaded boxes indicate the dates of spaying events for each of the treatments, the numerals indicate the number of sprays applied e.g. Control, NAA and Ethrel were only treated once. WA = wetting agent.

Bunches were treated until runoff using hand sprayers, except for the Control, for which a knapsack was used. The timing of the applications of the various reagents was designed to align with the stage of berry development predicted to be best suited to either promote or delay ripening. As it happened, the fruit were much more advanced than predicted which had implications for the effectiveness of the applications. AVG was applied multiple times as it is a competitive inhibitor of both ethylene biosynthesis and IAA biosynthesis, and needs to be present over a longer period to be effective, as it is readily transported through the plant which reduces its concentration at the treated site. ABA is quite labile, and a second treatment was thought to be required.

Berry samples of each of the treatment replicates were taken on various dates and kept on ice for transport to the lab for processing. The average berry weight was measured before the samples were

crushed and Brix measurements taken by digital refractometry and OenoFoss instrument conducted as required.

Final ('harvest') samples were taken for each treatment at equivalent Brix. For these samples, weight and Brix were measured as above and OenoFoss analysis conducted. From 11 to 17 kg of berries per replicate from Control and NAA-treated vines were harvested for small-scale wine production which was conducted by WIC Winemaking Services. Wine was not made from the other treatments as the delaying/advancing effect of the other treatments, as measured by berry weight and total soluble solids (TSS), measured as ^{o}Brix, was not sufficient to make this worthwhile.

6.1.3 Results/Discussion

6.1.3.1 Cabernet Sauvignon trial

The Cabernet Sauvignon vines were quite uneven with many vines having *Eutypa* symptoms e.g. some dead arm, affected shoots and in some cases vines were dead. This increased the complexity of the experiment as we had to try to choose panels that looked as healthy as possible and similar in yield and canopy size. The Shiraz vines were more even but again there were some vines that were affected and as with the Cabernet Sauvignon vines, we needed to find panels that were even in terms of canopy size and crop load.

Judging the time of veraison in blocks with which one has no previous experience can be challenging and this was the case here. Both the Shiraz and the Cabernet Sauvignon fruit commenced ripening earlier than anticipated as the estimated dates for veraison and harvest were later than those observed. As a result, the treatments were applied at later stages of berry development than we would have preferred and so the efficacy of all the treatments was not likely to be optimal. For example, NAA application was applied when at least some of the berries had commenced the ripening process, as seen by colour and berry softening. The ripening of berries cannot be delayed by auxin treatment once it has begun and so the NAA treatment at this stage may have increased asynchrony and reduced the overall (average) effect of NAA in delaying ripening. Because of the issues with application timing, the Ethrel 2 spray of Cabernet Sauvignon wines was omitted (see above). The timing of the other treatments was also not ideal and so this has most likely affected the final outcomes.

The mean berry weight for the fruit from all treatments, except those treated with NAA, decreased after reaching a maximum value at 80 days post flowering (dpf) (data not shown). In contrast, the NAA-treated fruit maintained a constant weight until harvest (Figure 4, for clarity only Control and NAA values are shown). It appears that the treatment somehow increased the ability of the berries to withstand the weight loss, which is presumably due to dehydration, which normally occurs during the later period of ripening. In a study outlined later in this report we have provided evidence that NAA treatment causes changes in the expression of cell wall modifying genes and in the cellular components that maintain cell wall integrity which may also influence cell viability. Stronger cells and less cell death would be important in maintaining berry integrity. In other experiments (see later in the report) we have seen indications that the rachis of Cabernet Sauvignon bunches maintains a healthier looking, greener appearance following NAA treatment and so the maintenance of rachis health could also be important for berry integrity.
The lack of berry shrinkage means that there is a benefit from NAA treatment in terms of yield. The average berry weight for NAA-treated fruit was 13.4% higher than the Control fruit. A large proportion of this increase in yield can be attributed to reduced shrivelling. This may represent a significant increase in crop yield/value.

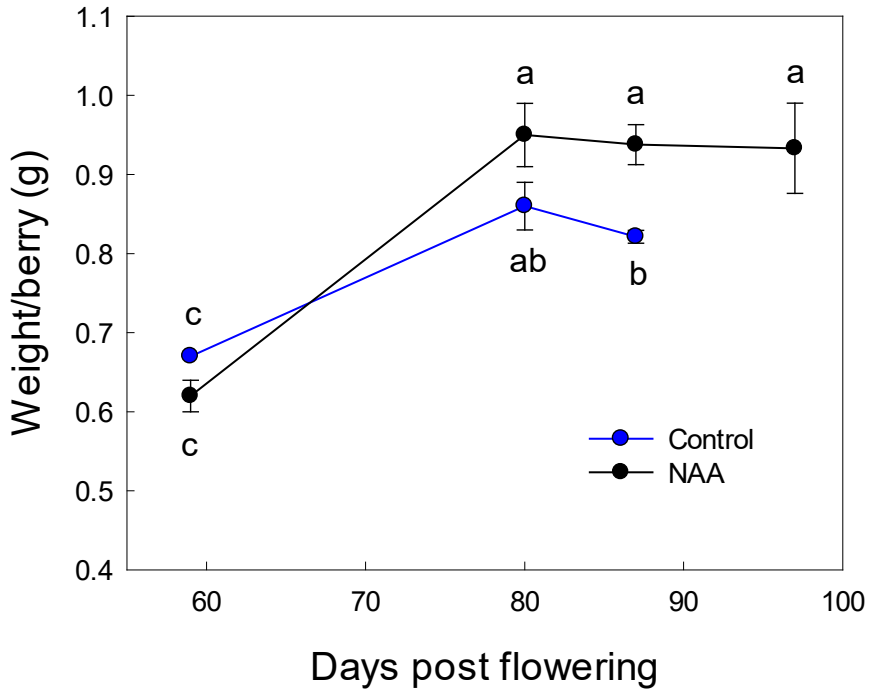


Figure 4. Mean berry weight during development for NAA-treated (black symbols) Cabernet Sauvignon fruit vs Control (blue symbols). Letters represent statistically significant groupings as determined by ANOVA analysis followed by Duncan's post hoc test, P<0.05

Sugar accumulation, as measured by increasing TSS (°Brix) was similar for Control, ABA, AVG and Ethrel-treated fruit (Figure 5) but was significantly delayed in NAA-treated fruit (Figure 5). Harvest for winemaking was 10 days later for NAA-treated fruit compared to the Control, although at harvest there was no significant difference in TSS between Control and NAA-treated fruit. This clearly indicates that NAA effectively delayed berry ripening. Longer delays in ripening have been observed in other studies (Böttcher *et al.,* 2011a, 2012a; Davies *et al.,* 2015) and they were probably due to more accurate timing of the spray, resulting in a more effective treatment. The 10 day delay is likely to be long enough to assist with winery intake at peak periods through spreading out the harvest period. When coupled with the lack of weight loss it could provide a greater yield at the desired time.

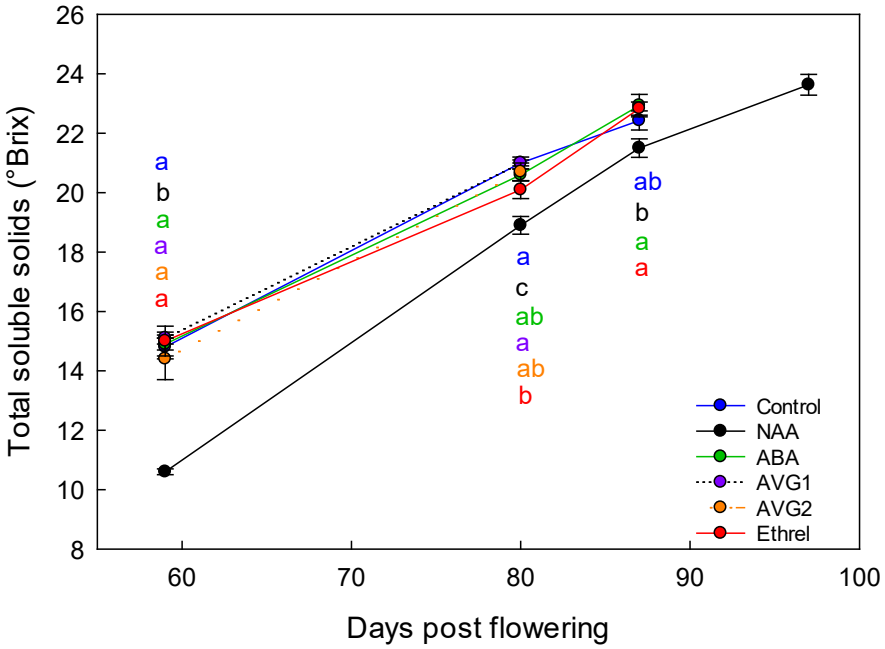
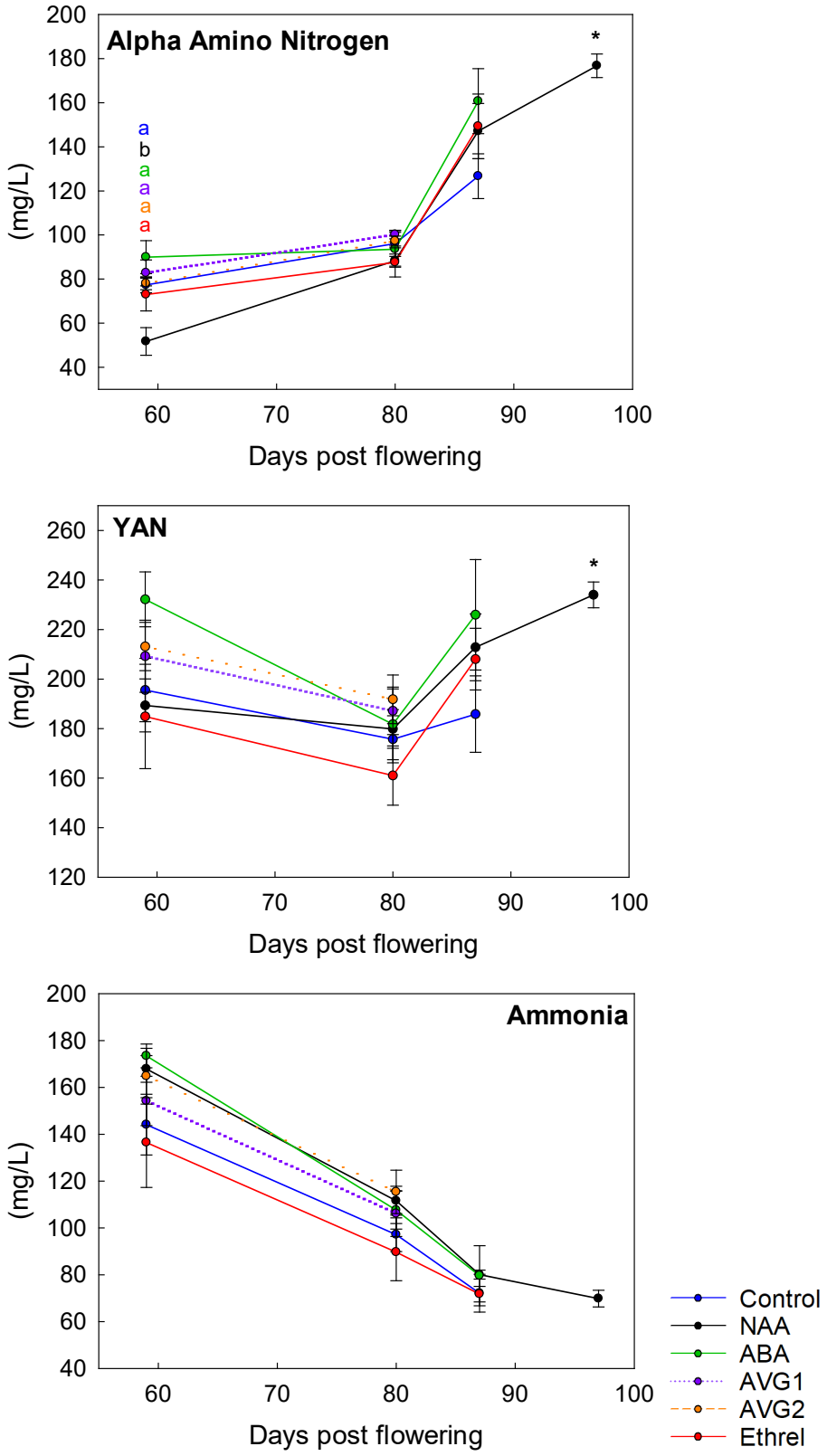
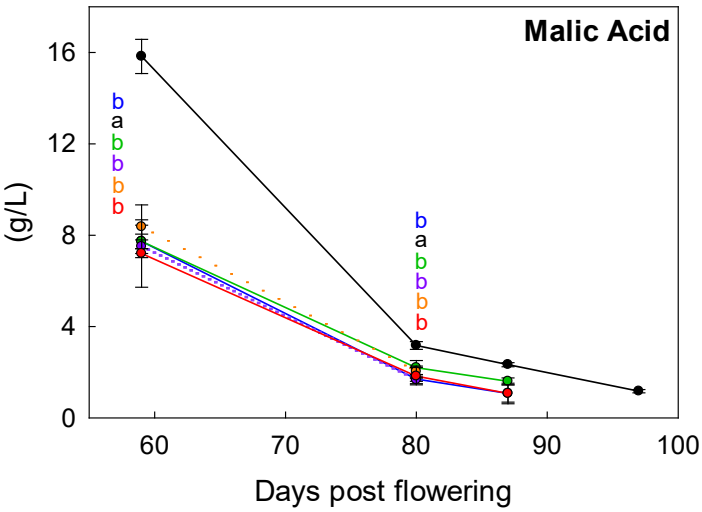
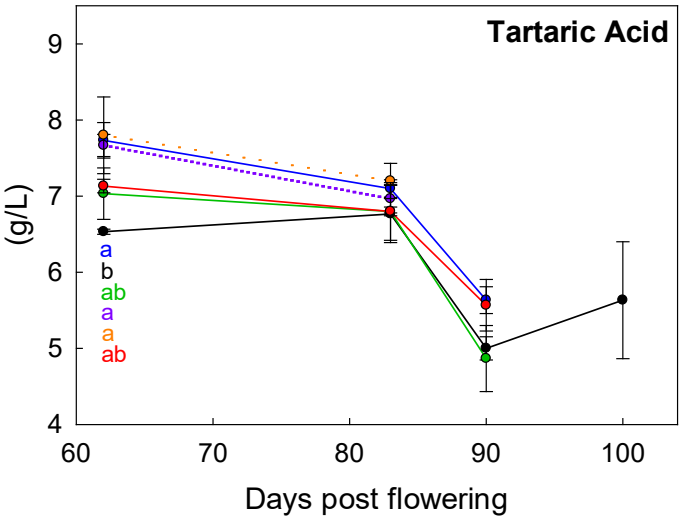


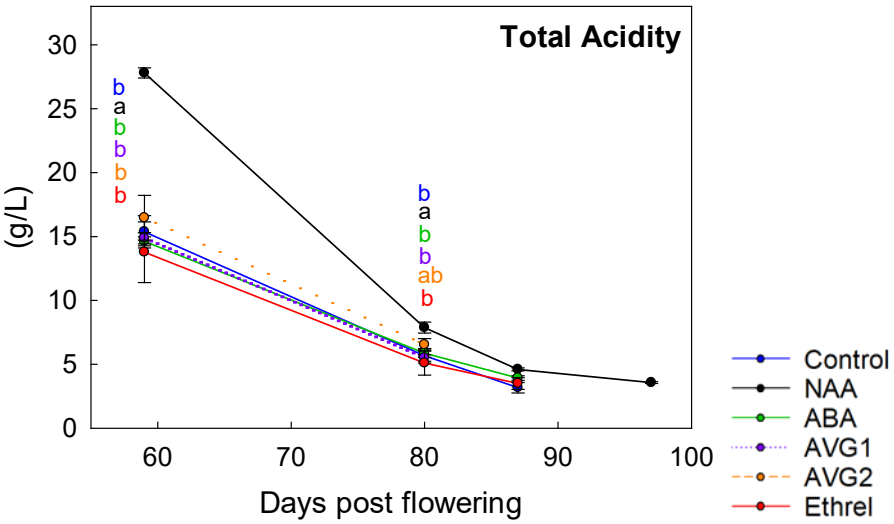
Figure 5. TSS (°Brix) during development for Control (blue), NAA (black), ABA (green), AVG (1 = purple, 2 = orange) and Ethrel-treated (red) Cabernet Sauvignon fruit. Letters represent statistically significant groupings as determined by ANOVA analysis followed by Duncan's post hoc test $P<0.05$.

Fourier transform infrared spectroscopy was used to measure various berry compositional parameters (Figure 6). The patterns were similar for Control, ABA, AVG and Ethrel-treated fruit. At harvest only alpha amino nitrogen, yeast assimilable nitrogen and pH were significantly different between Control and NAA-treated fruit. All were a little higher in NAA-treated fruit. It is interesting to note that the decrease in malic acid and total acidity that occurs in Control fruit during development is delayed in NAA-treated fruit (Figure 6) along with the delay in sugar accumulation (Figure 5).


Alpha Amino Nitrogen
*
a
b
a
a
a
a
(mg/L)
Days post flowering
YAN
*
(mg/L)
Days post flowering
Ammonia
(mg/L)
Days post flowering
Control
NAA
ABA
AVG1
AVG2
Ethrel


Malic Acid
(g/L)
Days post flowering
b
a
b
b
b
b
b
a
b
b
b
b


Tartaric Acid
(g/L)
Days post flowering
a
b
ab
a
a
ab


Total Acidity
(g/L)
Days post flowering
b
a
b
b
b
b
b
a
b
b
ab
b
Control
NAA
ABA
AVG1
AVG2
Ethrel

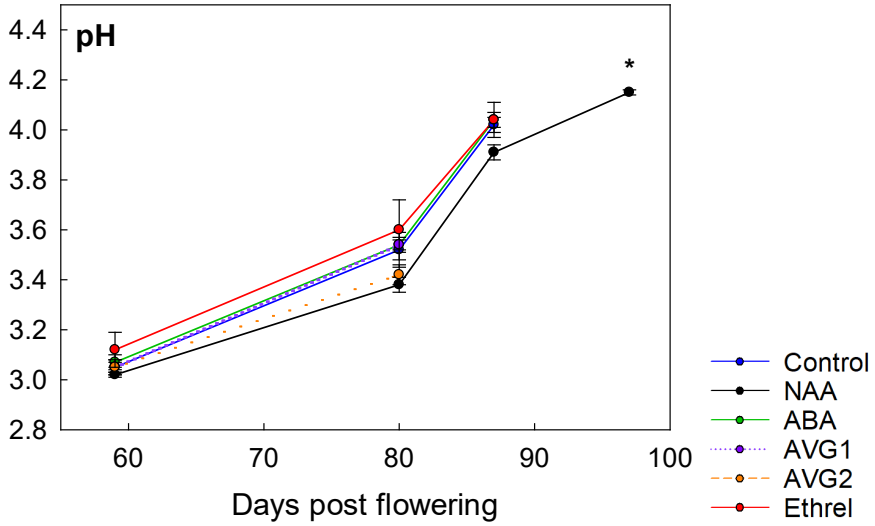


Figure 6. FTIR measurement of various berry parameters, alpha amino nitrogen, ammonia, tartaric acid, total acidity, malic acid, pH and YAN (yeast assimilable nitrogen) in Cabernet Sauvignon fruit. Various Letters represent statistically significant groupings as determined by ANOVA analysis followed by Duncan's post hoc test. The asterisks indicate a significant difference between Control and NAA-treated fruit at harvest as determined by t-test P<0.05.

Total soluble solids (°Brix) multiplied by average berry weight is a reasonable estimate of how much photosynthate (carbon) is stored per berry. Figure 7 shows the plot for Brix multiplied by average berry weight for Control and NAA-treated fruit. NAA-treated fruit stored more carbon on average than the Control. The NAA-treated fruit had slightly higher (no significant difference) Brix than the Control fruit at harvest (Figure 5), but the trend is clear that NAA treatment results in more carbon (as hexose sugars) being captured. As only bunches were sprayed in this experiment it seems unlikely that this is a result of a direct effect on leaf photosynthesis but rather an effect on berry metabolism.

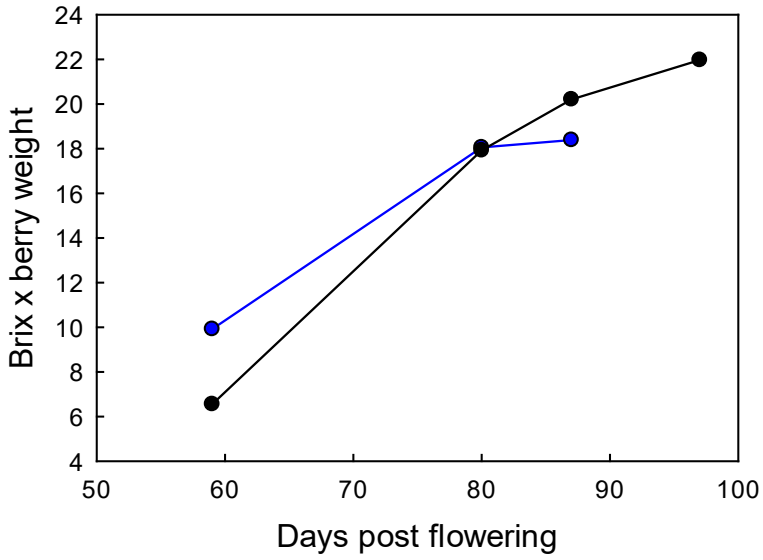


Figure 7. Total soluble solids (°Brix) x average berry weight for Control (blue symbols) and NAA (black symbols) Cabernet Sauvignon berries as a function of time (days post flowering).

NAA residues were measured in berries by LC-MS using the method described above. As can be seen from Figure 8 there were low levels of residual NAA in the NAA-treated fruit at harvest. These levels are very low and are below the detection levels used by an accredited laboratory that was recently contracted to conduct tests in line with Australian Pesticides and Veterinary Medicines Authority (APVMA) standards on similar material (Table 23).

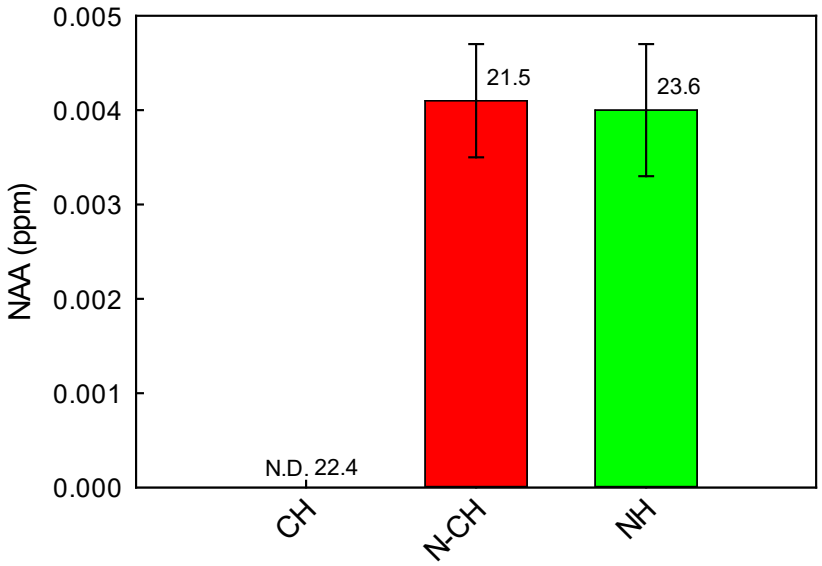


Figure 8. Concentration in ppm (mg/L) of NAA residues in Control fruit at harvest (CH) NAA-treated fruit at time of Control harvest (N-CH) and in NAA-treated fruit harvested at a similar Brix to the Control (NH), Brix values are indicated above the columns.

Small-scale wines were made by the WIC Winemaking Service from the three replicates of Control and NAA-treated fruit (six wines). As the berries from the other treatments showed little difference to the Control fruit with regard to berry developmental stage, it was decided to only make wine from the Control fruit and the NAA-treated fruit.

The winery crush data for Cabernet Sauvignon fruit showed that the yeast assimilable nitrogen (YAN) and alpha amino nitrogen (AAN) content was significantly higher in NAA-treated fruit, no other parameters were significantly different (Table 1). This confirms the data collected through berry development (Figure 6).

Table 1. Measurements for Control and NAA-treated fruit at crushing. TA = titratable acidity to pH 7 (g/L tartaric acid), malic acid (g/L), YAN = yeast assimilable nitrogen (mg/L), AAN = alpha amino acid nitrogen (mg/L), NH_3 ammonia (mg/L). The asterisks represent a significant difference between Control and NAA-treated fruit. T-test $P<0.05$

Crush Data Cabernet Sauvignon								
Date	**Treatment**	**Baume**	**pH**	**T.A.**	**Malic acid**	**YAN**	**AAN**	**NH3**
17 Feb	Control	12.73	3.51	4.30	2.25	99*	50*	60
26 Feb	NAA	12.57	3.58	4.47	2.49	170	108	74

Measurement of the concentration of anthocyanins in berries at harvest showed that although ABA and NAA treatments had no significant effect on total anthocyanin levels, Ethrel did show a significant increase compared with the Control.

Table 2. Anthocyanins (OD 520 nm) measured in berries at harvest by spectrophotometer. Each treatment was compared relative to the Control. Statistical significance was determined using Dunnett's t-test with post-hoc analysis, P<0.05

Treatment	Cabernet Sauvignon	Significant Difference vs Control
Control	47.69	-
ABA	51.56	No
Ethrel	62.58	Increased
NAA	47.49	No

As the alcohol level was significantly higher in the wines from Control fruit (Table 3) it might be assumed that the Control fruit was slightly riper than the NAA-treated fruit (as suggested by the trend in the Baume data at crush (Table 2)). The only other significant difference was the pH, which was a little higher in the wine from NAA-treated fruit but the pH adjustment during winemaking had obviously worked well. NAA residue in the bottled wine was 0.05 ppm.

Table 3. Measurements for Control and NAA-treated fruit at the pre-bottling stage. Glc+Fru = glucose and fructose content (g/L), SG = specific gravity (g/mL), TA7, TA8.2 = titratable acidity to pH7 and 8.2 respectively (g/L), malic acid (g/L), VA = volatile acidity (g/L). The asterisks represent a significant difference between Control and NAA-treated fruit. T-test P<0.05

Pre-bottle Data Cabernet Sauvignon								
Treatment	**Alcohol (%)**	**Glc+Fru**	**S.G.**	**pH**	**TA7**	**TA8.2**	**Malic acid**	**VA**
Control	13.50*	0.63	0.9933*	3.55*	5.8	6.4	0.06	0.48
NAA	13.03	0.50	0.9937	3.60	5.8	6.5	0.06	0.47

6.1.3.2 Shiraz trial

The effects of the various treatments on Shiraz berry weight followed a similar pattern to that observed for Cabernet Sauvignon. The mean weight of all the berries except for those treated with NAA decreased after reaching a maximum average weight at 83 dpf (Figure 9 and data not shown). Again, the NAA-treated berries had a higher average harvest weight, 14.5% higher than Control berries at harvest as they maintained their weight after 83 dpf and did not shrink appreciably. The processes involved appear to be similar to those discussed above for Cabernet Sauvignon and represent a significant increase in harvest yield.

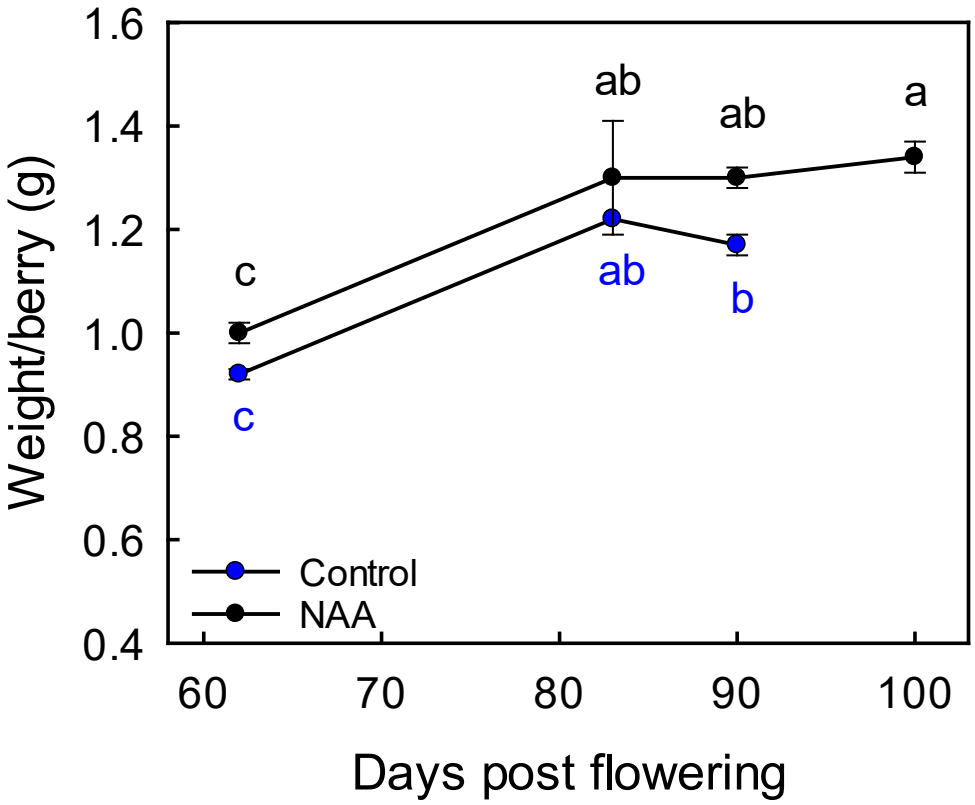


Figure 9. Mean berry weight during development for NAA-treated (black) Shiraz vs Control fruit (blue). Letters represent statistically significant groupings as determined by ANOVA analysis followed by Duncan's post hoc test, $P<0.05$.

The pattern of sugar accumulation, as measured by increasing TSS (^{o}Brix) was similar to that seen for Cabernet Sauvignon fruit (compare Figures 5, 10). The increase in TSS had a similar pattern in Control, ABA, AVG and Ethrel-treated fruit but was significantly delayed in NAA-treated fruit (Figure 10). Harvest for winemaking was 10 days later for NAA-treated fruit compared to the Control, and at harvest there was no significant difference in TSS. Again, this showed that NAA-treatment can delay berry ripening in a way that should be useful to industry interested in spreading the harvest season over a longer period.

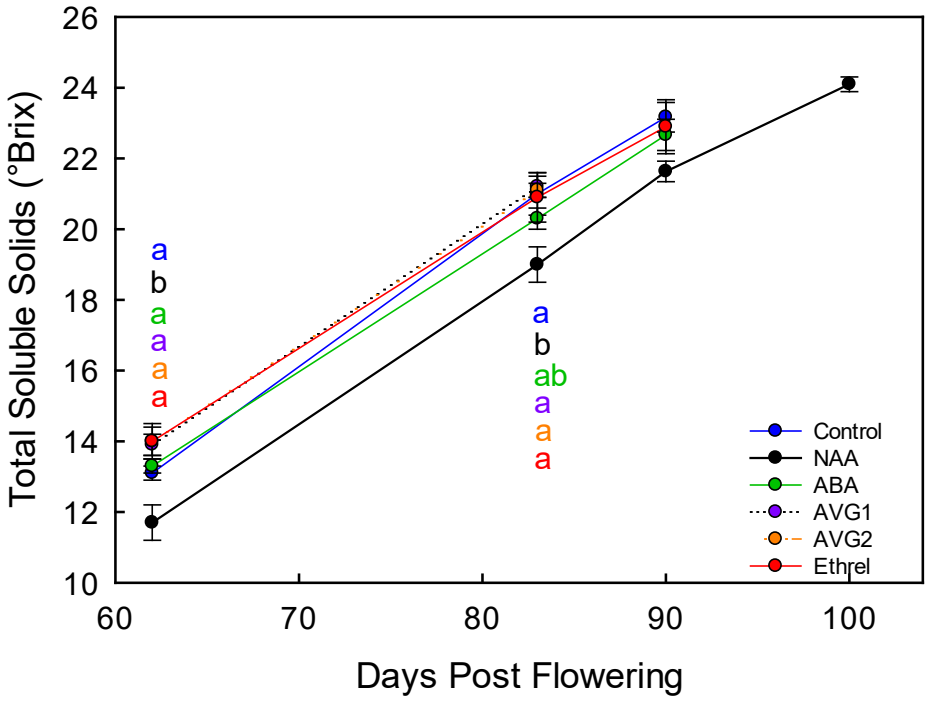
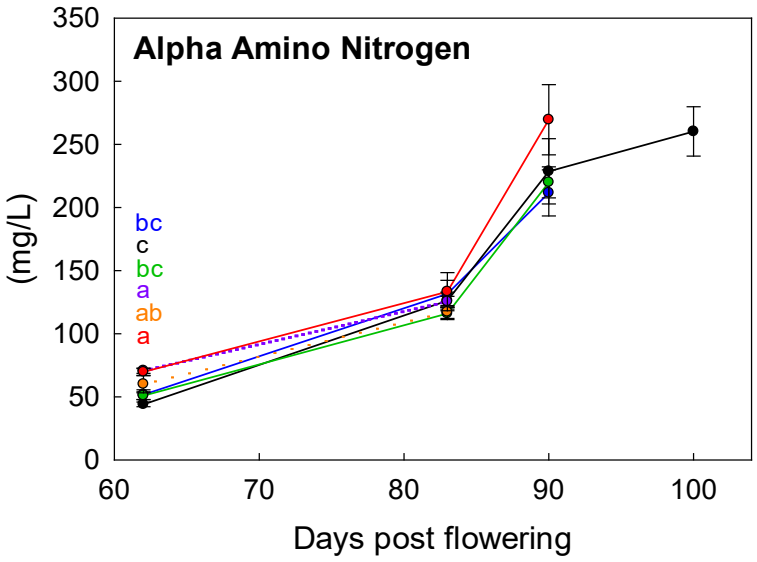
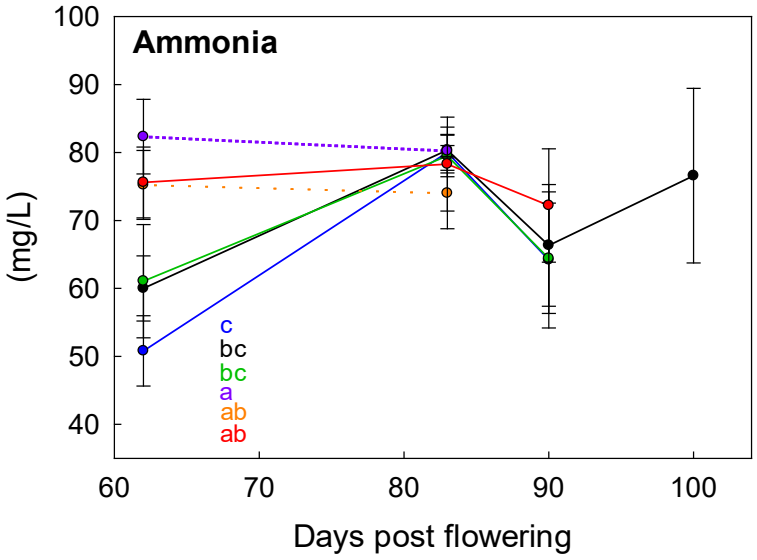
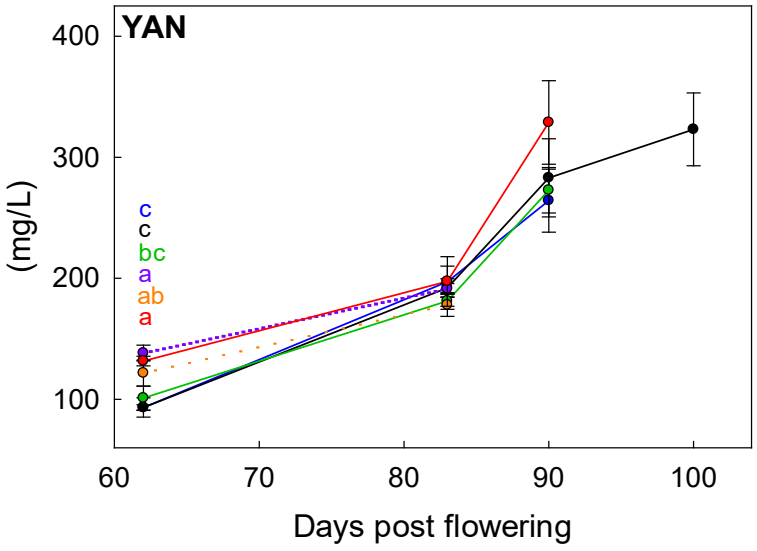


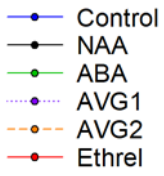
Figure 10. TSS (°Brix) during development for Control (blue), NAA (black), ABA (green), AVG (1 = purple, 2 = orange) and Ethrel-treated (red) Shiraz fruit. Letters represent statistically significant groupings as determined by ANOVA analysis followed by Duncan's post hoc test, $P<0.05$.

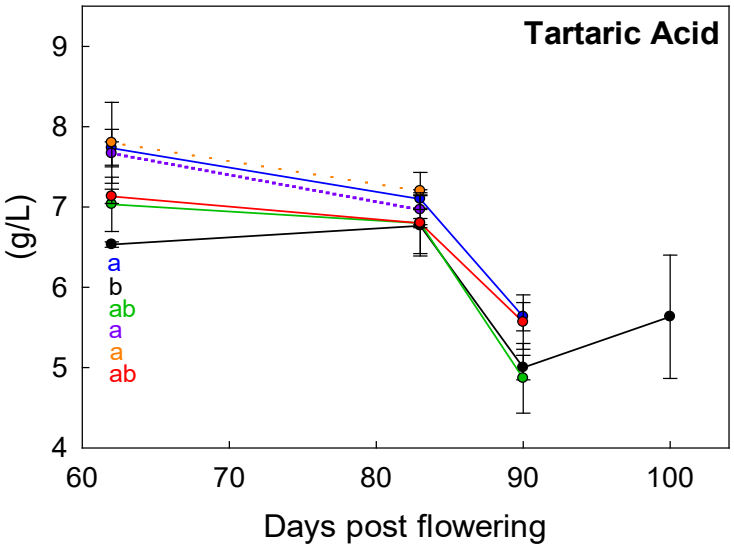
Fourier transform infrared spectroscopy was used to measure various berry composition parameters (Figure 11). The patterns were similar for Control, ABA, AVG and Ethrel-treated fruit. At harvest only malic acid and total acidity were lower for NAA-treated fruit; the pH was not significantly different.

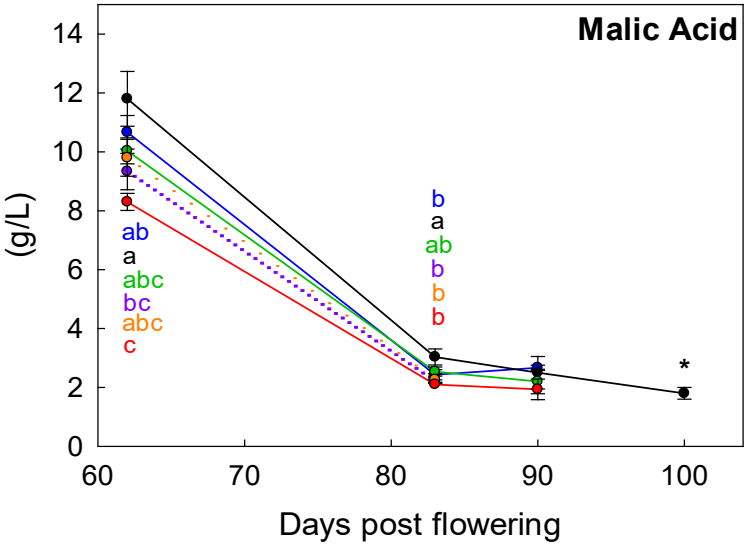

Alpha Amino Nitrogen
(mg/L)
Days post flowering
bc
c
bc
a
ab
a

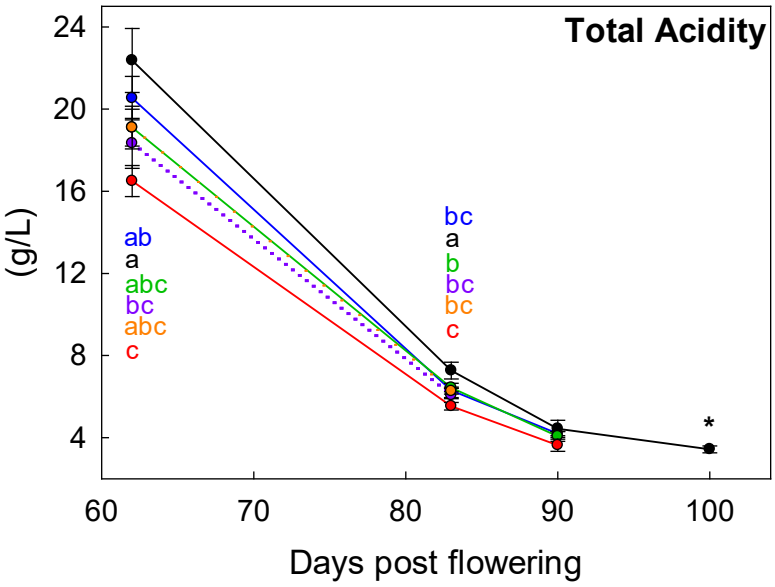

Ammonia
(mg/L)
Days post flowering
c
bc
bc
a
ab
ab


YAN
(mg/L)
Days post flowering
c
c
bc
a
ab
a


Control
NAA
ABA
AVG1
AVG2
Ethrel


Tartaric Acid
(g/L)
Days post flowering
a
b
ab
a
a
ab


Malic Acid
(g/L)
Days post flowering
ab
a
abc
bc
abc
c
b
a
ab
b
b
b
*


Total Acidity
(g/L)
Days post flowering
ab
a
abc
bc
abc
c
bc
a
b
bc
bc
c
*
Control
NAA
ABA
AVG1
AVG2
Ethrel

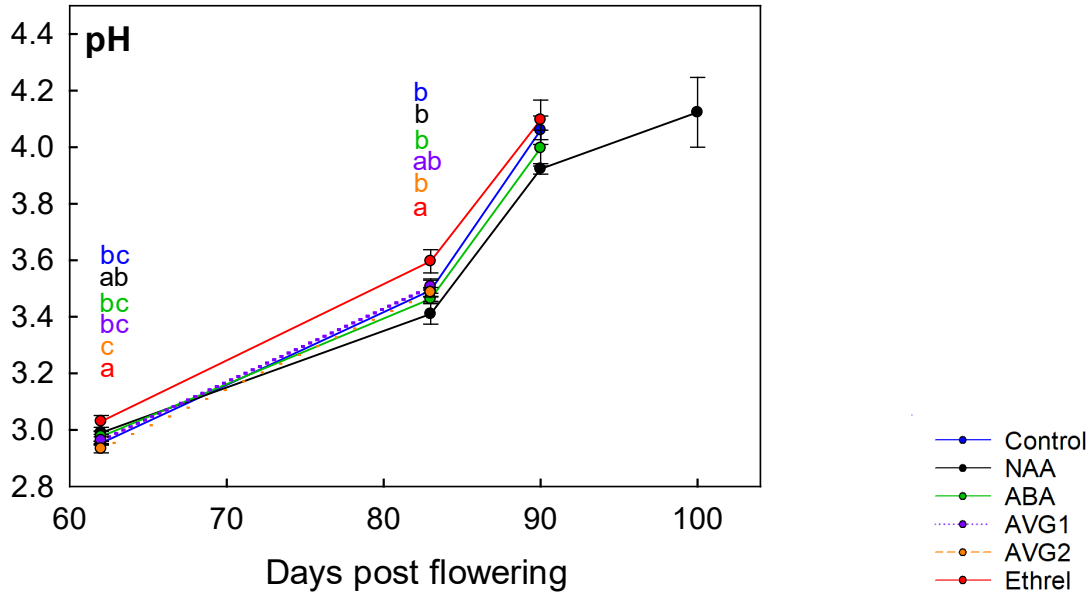


Figure 11. OenoFoss (FTIR) measurement of various berry parameters, alpha amino nitrogen, ammonia, tartaric acid, total acidity, malic acid, pH and YAN (yeast assimilable nitrogen). Letters represent statistically significant groupings as determined by ANOVA analysis followed by Duncan's post hoc test. The asterisks indicate a significant difference between Control and NAA-treated fruit at harvest as determined by t-test, $P<0.05$.

The pattern for the Brix x average berry weight plot for Control and NAA-treated Shiraz berries (Figure 12) is similar to that for Cabernet Sauvignon (Figure 7) as although the NAA-treated fruit was a little riper, having a higher Brix value, the NAA-treated fruit shrivelled much less than the Control berries. Again it is clear that the NAA-treated berries stored considerably more photosynthate than the Control fruit.

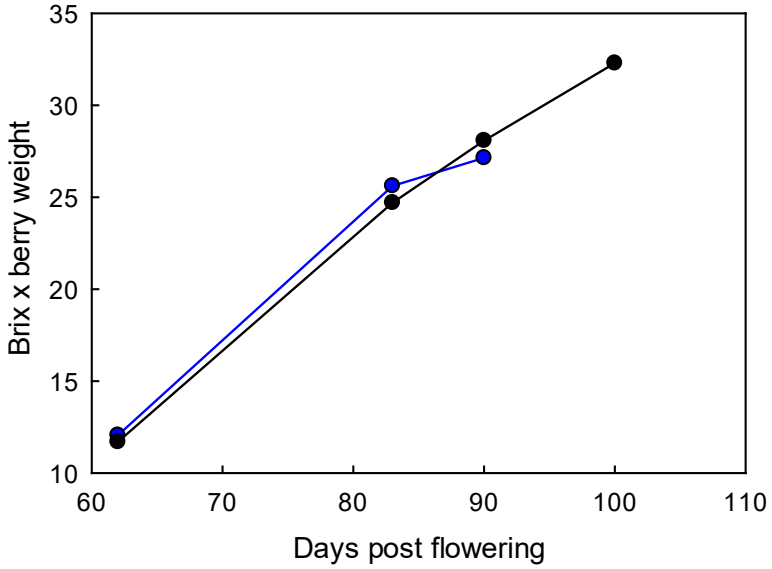


Figure 12. Brix (TSS) x average berry weight for Control (blue symbols) and NAA (black symbols) Shiraz berries as a function of time (days post flowering).

The measurement of NAA residues in the NAA-treated Shiraz fruit showed that the berries contained approximately 0.004 ppm NAA at the time of the harvest of the Control (N-CH) and approximately half as much at harvest (Figure 13). The decrease at the NH stage was not observed for Cabernet Sauvignon but the levels detected are very low and as mentioned above for Cabernet, they are below detectable levels.

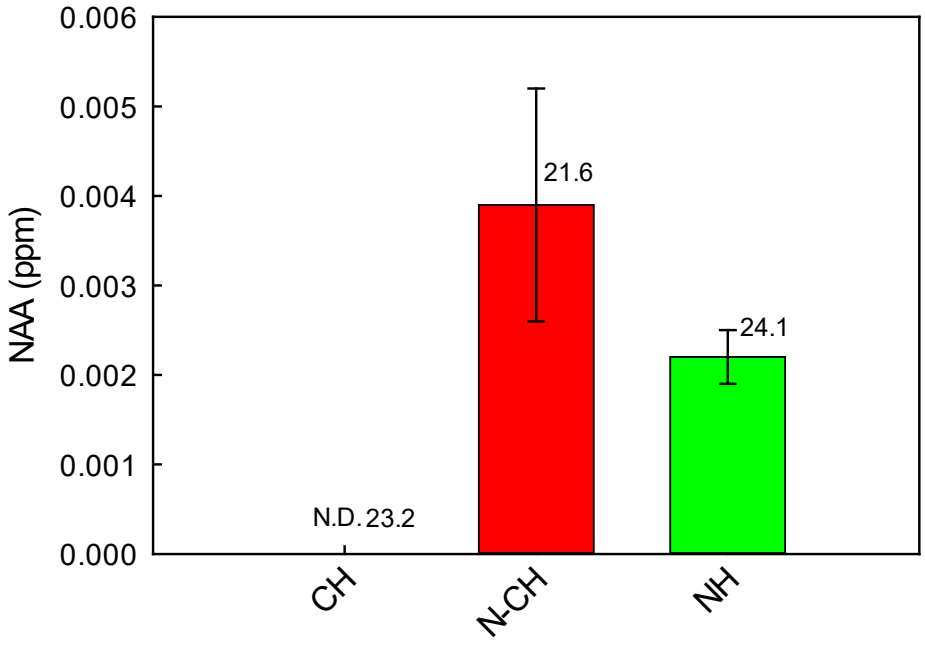


Figure 13. Concentration in ppm (mg/L) of NAA residues in Control fruit at harvest (CH) NAA-treated fruit at time of Control harvest (N-CH) and at NAA-treated fruit harvest (NH). Brix values are indicated above the columns.

Small-scale wines were made by the WIC Winemaking Service from the three replicates of Control and NAA-treated fruit (six wines). As reported for Cabernet Sauvignon above, the berries from the other treatments showed little difference to the Control fruit with regard to berry developmental stage and therefore wine was only made from the Control fruit and the NAA-treated fruit.

Measurement of berry composition at crush indicated that the pH of the NAA-treated fruit was significantly higher than that of Control berries (Table 4). In contrast, no significant difference was observed for Cabernet Sauvignon (Table 1). The reason for the differences in pH between the two cultivars is not known. As for Cabernet YAN and AAN were both significantly higher in NAA-treated fruit.

Table 4. Measurements for Control and NAA-treated fruit at crushing. TA = titratable acidity to pH 7 (g/L tartaric acid), malic acid (g/L), YAN = yeast assimilable nitrogen (mg/L), AAN = alpha amino acid nitrogen (mg/L), NH_3 ammonia (mg/L). The asterisks represent a significant difference between Control and NAA-treated fruit. T-test $P<0.05$

Crush data for Shiraz								
Date	**Treatment**	**Baume**	**pH**	**TA**	**Malic acid**	**YAN**	**AAN**	**NH_3**
17 Feb	Control	12.73	3.57*	4.13	2.66	177*	136*	50
26 Feb	NAA	13.17	3.87	3.83	2.55	247	200	57

Total anthocyanins were measured in Control and NAA-, ABA- and Ethrel-treated fruit at harvest (when at similar Brix) (Table 5). None of the treatments significantly affected anthocyanin accumulation.

Table 5. Anthocyanins (OD 520 nm) measured in berries at harvest by spectrophotometer. Each treatment was compared relative to the Control. Statistical significance was determined using Dunnett's t-test with post-hoc analysis, $P<0.05$

Treatment	**Shiraz**	**Significant Difference vs Control**
Control	50.20	-
ABA	51.98	No
Ethrel	60.05	No
NAA	58.67	No

At the pre-bottling stage the only difference between wines from Control and NAA-treated fruit was that the wine from NAA-treated fruit had a slightly higher pH (Table 6), indicating that the pH adjustment was a little inaccurate. As all the other parameters were similar, it seems that the basic structure of the wine is very similar. The NAA residue in bottled wine was measured at 0.04 ppm.

Table 6. Measurements for Control and NAA-treated fruit at the pre-bottling stage. Glc+Fru = glucose and fructose content (g/L), SG = specific gravity (g/mL), TA7, TA8.2 = titratable acidity to pH7 and 8.2 respectively (g/L), malic acid (g/L), VA = volatile acidity (g/L). The asterisk represents a significant difference between Control and NAA-treated fruit. T-test P<0.05

Pre-bottle Data Shiraz								
Treatment	**Alcohol (%)**	**Glc+Fru**	**SG**	**pH**	**TA7**	**TA8.2**	**Malic acid**	**VA**
Control	13.70	0.63	0.9930	3.58*	5.4	6.1	<LOQ	0.38
NAA	13.87	0.93	0.9932	3.54	5.7	6.3	0.05	0.40

6.1.3.3 Wine sensory properties

The sensory properties of the Control and NAA-treated wines from both Cabernet Sauvignon and Shiraz vines were evaluated by an expert panel. In a blind tasting, the three replicates for each treatment were compared against each other to identify outliers and then the Control and NAA-treated wines were compared for each of the cultivars.

Cabernet Sauvignon
The wines from the NAA-treated vines were preferred over the Control wines. In terms of aroma the Control wines were described as 'dusty', 'baggy' and 'herbal' with 'green' notes. In contrast, the wines from NAA-treated fruit were described as having 'dark fruit', 'minty' and even 'medicinal' aromas. On the palate the Control wines were seen to be 'thin', having 'red fruit notes' with some 'dark fruit, medicinal and mint' but less accented than in the NAA wines. The NAA wines tasted more strongly of 'dark fruit' again with some 'red fruit' but with some 'medicinal' qualities and a fuller body overall.

Shiraz
As with the Cabernet Sauvignon wines, the wines from NAA-treated berries were preferred to those from Control vines. No peppery notes were detected in any of the six wines. The aroma of the Control wines had a somewhat unpleasant aroma being variously described as 'green', 'oyster shell' with some 'dark confection' and one panellist detected hints of 'stone fruit'. The wines from delayed ripening, NAA-treated fruit had aromas of 'dark fruit' with some 'red fruit', 'plummy', 'floral', 'confectionary' notes and were described as seeming 'fresher'. On the palate the Control wines were described as 'thin' but having some 'red fruit' and 'floral' notes. Again, in contrast, the wines from NAA-treated fruit were described as being 'fuller bodied' with 'plummy', 'dark fruit' and 'floral' characters.

Overall, the wines from fruit of both cultivars that had been delayed in ripening by NAA treatment were preferred over the non-delayed (untreated), Controls. The wines harvested at the later date but at the same Brix were seen as being riper having more 'fruity' and less 'green' characters.

6.1.4 Conclusions

The treatment timing used in this study was not as accurate as we would have liked, due to unfamiliarity with the two selected trial sites. This shows the importance of application timing and the need to be able to monitor berry development at regular intervals, particularly in the weeks leading up to veraison. In addition, the stages of development in these vineyards were somewhat asynchronous, which may have also detracted from the resolution of the study. This is compounded by the constraints of modestly sized trials that had to be hand-treated. However, although this may have affected the success of treatments such as ABA, AVG and Ethrel, it confirmed that NAA treatment is quite robust and effective even under less-than-optimal conditions. NAA treatment still delayed harvest, as measured by TSS, in both Cabernet

Sauvignon and Shiraz and the delay was long enough that it could be useful to industry in spreading out a compressed harvest season. Interestingly, NAA treatment produced berries with a higher mean weight than that of Control fruit. This was partly due to the NAA-treated berries reaching a slightly higher maximum weight but mainly because neither the Cabernet Sauvignon nor Shiraz fruit lost weight appreciably during the last weeks of ripening compared to the weight loss observed in the Control fruit. The reasons for this are not known but some results from our other experiments suggest that NAA treatment could work via two mechanisms. One reason is that the NAA-treated berries of both Cabernet Sauvignon and Shiraz appeared to be in better condition at harvest than Control fruit. We know from other work (below) that NAA causes changes to cell walls that could make them more stable. Secondly, NAA seems to maintain rachis 'health' (see below) and this may also help by maintaining phloem conductivity with the berry. As the NAA-treated berries were larger but at the same Brix at harvest, it means that they stored more photosynthate than Control berries. This increase in yield is another benefit for growers. At crush, both YAN and AAN were higher in NAA-treated fruit. There is no obvious explanation for this but having YAN somewhat higher may help to prevent stuck ferments, which would be an advantage for winemakers.

Tasting of the wines from Control and NAA-treated fruit showed that the wines that had been delayed in harvest date through NAA treatment were preferred, even though they were harvested at the same Brix, albeit later in the season. This difference could be due to the extension of the pre-veraison period in the NAA-treated grapes, which may have given extra time for the production of favourable aroma compounds and their precursors. It could also have been due to direct effects of NAA on berry metabolism, which may have led to differences in wine character. Another, perhaps not so obvious effect could be that we have observed over a number of experiments that NAA treatment seems to better preserve the health of the rachis and the integrity of berries during the later stages of ripening, which could be due to more stable cell wall structure (Section 6.4). Improved rachis and berry health and integrity may improve flavour and aroma outcomes.

6.2 **Use of auxin to delay ripening – sensory and biochemical evaluation of Cabernet Sauvignon and Shiraz**

6.2.1 Introduction

The aim of this study was to further investigate NAA application as a treatment to delay ripening/harvest by comparing the effects on the two most commonly grown red grape cultivars grown in Australia i.e. Shiraz and Cabernet Sauvignon. The trial described in Section 6.1 indicated that NAA was likely to be more reliable than the other treatments used in altering the timing of ripening/harvest but the trial conditions, partly due to the necessity to reduce the scale of each individual treatment to make it manageable, were thought not to be a true test of the potential for NAA to delay ripening. The study described here focused solely on a comparison between Control and NAA treatments. Apart from testing the potential of NAA to delay harvest, its effect on grape development and on grape and wine composition and sensory properties were investigated.

6.2.2 Materials and methods

6.2.2.1 Auxin treatment of field-grown wine grapes

Bunches from either *Vitis vinifera* L. cv. Cabernet Sauvignon or cv. Shiraz vines, grown at two different sites, were sprayed to runoff with 100 mg/L NAA (Gibco BRL Life Technologies, Grand Island, USA) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, Australia). Control fruit were sprayed with a 0.1% (v/v) Chemwet 1000 solution. The Cabernet Sauvignon vines were located at a drip-irrigated, commercial vineyard at Padthaway, South Australia and were treated twice, on 12 January (41 days post-flowering (dpf) and 22 days pre-veraison) and 18 January (47 dpf and 16 days pre-veraison). The Shiraz vines were located at a drip-irrigated, commercial vineyard at Eden Valley, South Australia and were treated once, 31 January (53 dpf, nine days pre-veraison). Both trials were of a randomised triplicate design and the sample size per replicate was 700 bunches. At regular intervals during development, berry samples were collected for the measurement of basic fruit quality parameters, anthocyanins, plant nutrients and selected aroma compounds as described below.

6.2.2.2 Determination of fruit weight, total soluble solid (TSS) level

At regular intervals starting from 41 dpf (Cabernet Sauvignon) or 47 dpf (Shiraz), 100 berries per replicate were randomly sampled into plastic bags, which were sealed, transported to the laboratory within 2 h of sampling and weighed. Individual TSS measurements were taken for each berry using a digital refractometer and averaged across each replicate to obtain average berry TSS values. A second subset of 60 berries (Cabernet Sauvignon, starting at 41 dpf) or 60 g of berries (Shiraz, starting at 25 dpf) was immediately frozen in liquid nitrogen and stored at -80°C.

6.2.2.3 Small-scale winemaking

Control and NAA-treated fruit were harvested at a TSS level of 24-25 ˚Brix, which resulted in the following harvest dates for Shiraz: Control, 21 April; NAA, 15 May and Cabernet Sauvignon: Control, 19 April; NAA, 10 May. Fifty kg of fruit for each of the three replicates from each treatment was used for small-scale winemaking by WIC Winemaking Services, with the addition of malolactic fermentation.

6.2.2.4 Rate-All-That-Apply (RATA) sensory evaluation of small-scale wines

Sensory profiles of the wines were generated by RATA analysis (Danner *et al.,* 2018) at the University of Adelaide sensory laboratory, which complies with international standards for the design of test rooms (ISO 8589:1988). Prior to the formal RATA sessions, a panel consisting of five wine experts assessed the 12 wines for any faults and developed a RATA attribute list based on generic red wine sensory attribute lists described in previous studies (Crump *et al.,* 2015; Danner *et al.,* 2018) with the removal of all colour and oak related attributes. This resulted in a RATA attribute list consisting of 18 aroma and flavour, three taste, six mouthfeel and two aftertaste attributes.

The RATA analysis was performed with the six Shiraz wines evaluated by 60 assessors in the first week and the six Cabernet Sauvignon wines evaluated by 60 assessors in the following week. Assessors were represented by a mix of occasional and regular wine consumers, oenology and viticulture students, trained wine tasters and winemakers of varying ages. There was an overlap of 46 assessors between the Shiraz and the Cabernet Sauvignon sessions. The evaluations were undertaken in individual sensory booths at 23˚C under red light to remove any colour cue in the wine that might introduce bias. Wines (30 mL) were served individually in a randomised order in transparent ISO-standard wine glasses labelled with a four-digit code and covered with watch glasses. Assessors rated aroma followed by taste, flavour, mouthfeel and aftertaste of each sensory attribute that they perceived to be present on a 7-point scale as described by Danner *et al.* (2018). RATA data were collected using RedJade software (RedJade; Redwood City, USA) and analysed with Senpaq (ver. 5.01; QiStatistics, UK) and XLSTAT (ver. 2018; Addinsoft SARL, France).

6.2.3 Results and discussion

6.2.3.1 Effect of NAA treatment on ripening parameters and composition of Shiraz and Cabernet Sauvignon grapes

As with previous studies (Böttcher *et al.,* 2011a,b; Davies *et al.,* 2015) the delaying effect of pre-veraison NAA treatments on Shiraz berry growth, sugar accumulation and colour development was confirmed in this trial (Figure 14a-c). Sugar accumulation was the first of the three ripening parameters to be affected (Figure 14b), with reduced TSS values recorded in NAA-treated grapes from veraison of the Control fruit (nine days post-treatment) to harvest of the Control fruit. NAA-treated berries were harvested 24 days later when they had reached a similar TSS levels to the Control fruit. A significant delay in the post-veraison increase in berry weight after NAA treatment became apparent from 75 dpf (nine days post-veraison), but a higher growth rate of the NAA-treated grapes between 82 and 96 dpf meant that, at 96 dpf, Control and NAA-treated fruit both reached a comparable final berry weight (Figure 14a). Similarly, the initial rate of anthocyanin accumulation (66-96 dpf) was lower in NAA treated berries compared with the Control, but when harvested at the same TSS level there was no statistically significant difference in anthocyanin concentration between the treatments (Figure 14c).

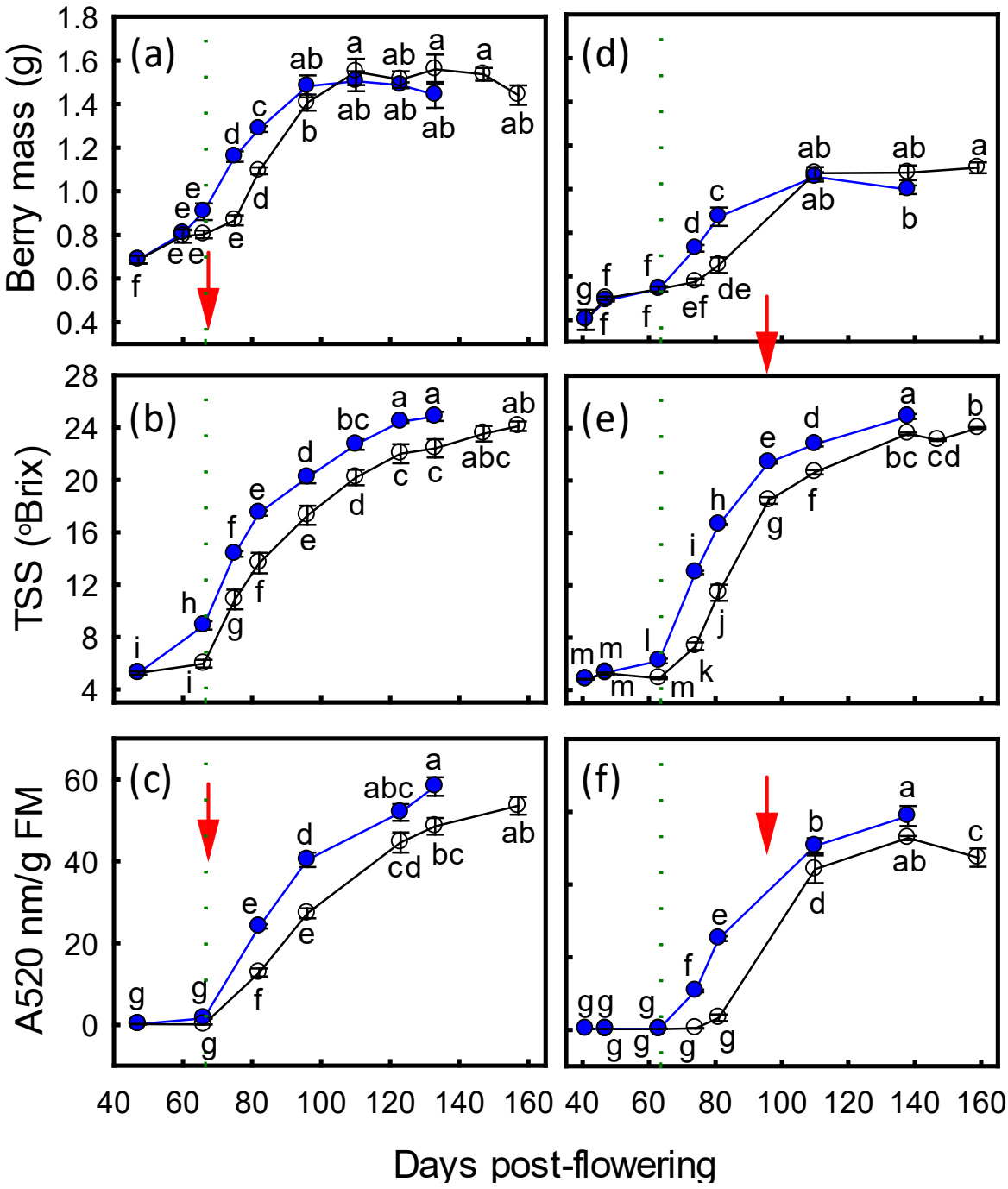


Figure 14. (a,d) Weight, (b,e) TSS and (c,f) anthocyanin (A520 nm) accumulation in developing Shiraz (a-c) and Cabernet Sauvignon (d-f) Control (●) and NAA-treated (○) berries. Red arrows indicate time of (initial) spray and dashed lines mark veraison of the Control fruit. Values represent means ± SE (n = 3) and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test. FM, fresh weight.

The delay in colour accumulation in NAA-treated fruit can be clearly observed in Figure 15 where 13 days after NAA treatment, the bunches of Control fruit had numerous coloured berries but the NAA-treated fruit showed no visible skin colouration.

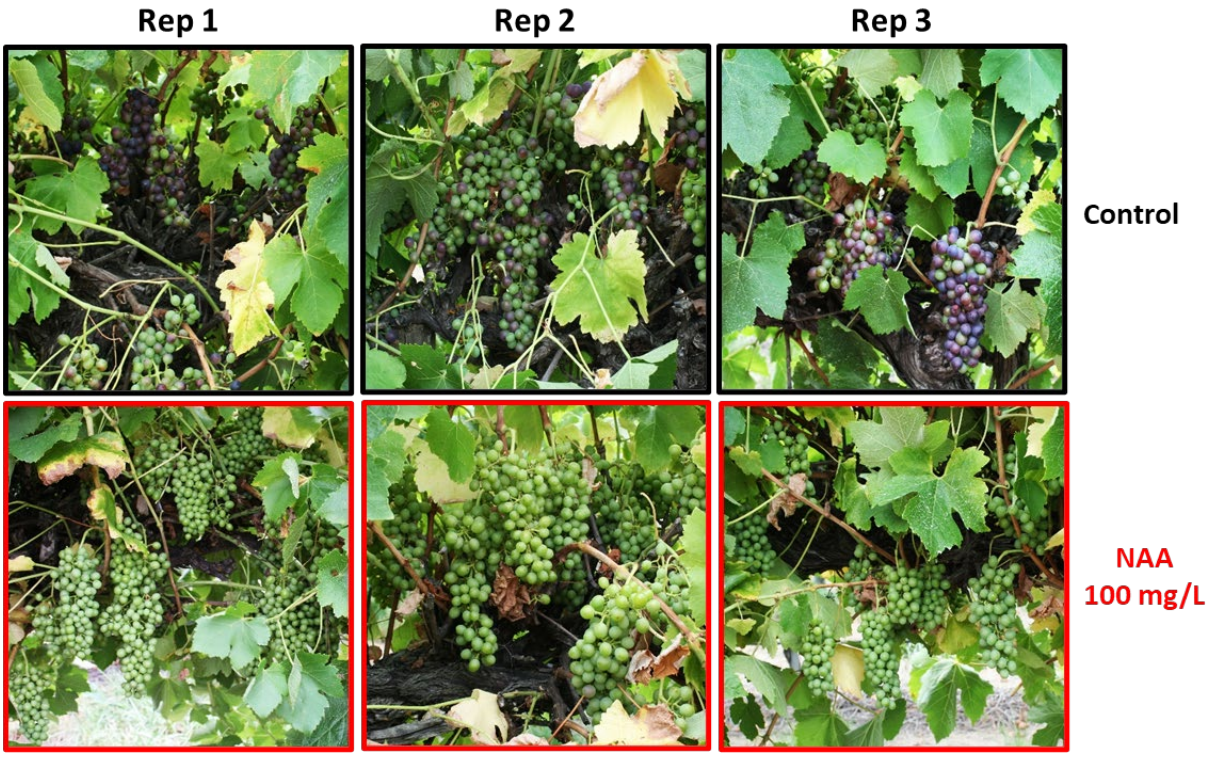


Figure 15. Typical examples of Shiraz vines treated with either wetting agent only (Control) or 100 mg/L NAA as per methods. Photographs were taken 13 days after single NAA treatment.

Despite differences in geographic location and treatment regime, Cabernet Sauvignon grapes responded to NAA applications in a very similar way to Shiraz grapes (Figure 14d-f). Berry weight, TSS and anthocyanin concentrations followed the same trajectories as for Shiraz described above however, NAA-treated Cabernet Sauvignon berries had significantly higher weight and significantly lower TSS and anthocyanin levels at harvest compared with the Control fruit, harvested 21 days earlier. A berry weight increase in response to NAA treatment has previously been observed in Shiraz (Böttcher *et al.,* 2011b) and could be a result of enhanced cell expansion (Dal Santo *et al.,* 2020), possibly related to the use of multiple pre-veraison applications and/or timing of the treatments. NAA fruit were also found to have slightly lower anthocyanin concentrations when compared to Control fruit at comparable harvest TSS levels (Böttcher *et al.,* 2011b). The reason for this NAA-induced reduction in anthocyanin accumulation is unknown.

The delay in colour accumulation observed in Shiraz is also clearly visible in photographs of Control and NAA-treated Cabernet Sauvignon bunches (Figure 16).

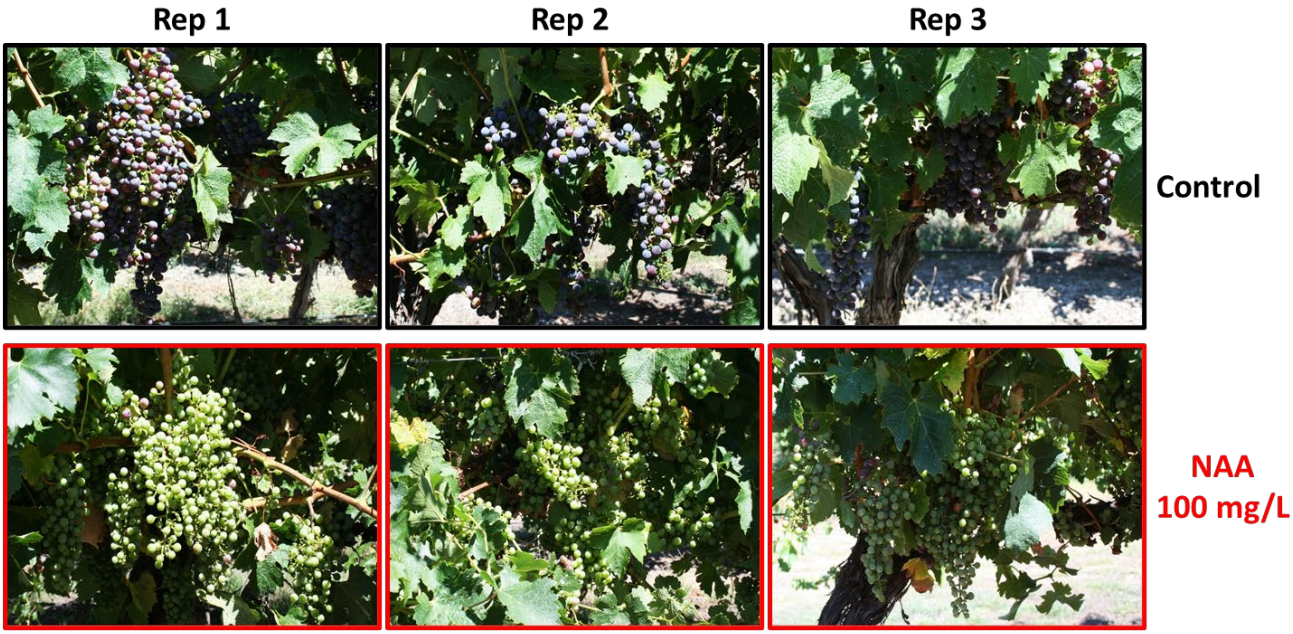


Figure 16. Typical examples of Cabernet Sauvignon vines treated with either wetting agent only (Control) or 100 mg/L NAA as per materials and methods. Photographs were taken 27 days after the second NAA treatment.

At the time of crushing during winemaking, Control and NAA-treated Shiraz and Cabernet Sauvignon fruit showed no significant difference ($P < 0.05$) in TSS, pH, titratable acidity, malic acid concentration and yeast assimilable nitrogen (YAN) between the two treatments for either cultivar (Table 7). It seems that the small difference in TSS between Cabernet Sauvignon Control and NAA-treated grapes at harvest described above (Figure 14e) was due to a minor variation in the subsamples that were taken for tracking the developmental progression of the berries.

Table 7. Composition of Control and NAA-treated Shiraz and Cabernet Sauvignon (CS) grapes.

Cultivar	Treatment	Crush data				
		TSS (°Brix)	pH	TA 8.2 (g/L)	Malic acid (g/L)	YAN (mg/L)
Shiraz	Control	24.37	3.78	3.37	2.00	194.33
	NAA	24.80	3.65	4.13	2.10	163.33
CS	Control	25.20	3.75	3.80	2.17	197.67
	NAA	24.47	3.74	3.97	2.03	177.67

Values are the mean of $n = 3$ biological replicates. TA, titratable acidity; YAN, yeast-available nitrogen.

The macro- and micronutrient composition of the grapes of both cultivars was also largely unaffected by the NAA treatment, at harvest and throughout development as the only elements with significantly different concentrations at two or more time points during development were calcium and potassium in Cabernet Sauvignon grapes (Figure 17).

Calcium is a xylem-mobile element that is known to accumulate in pre-veraison grapes (Rogiers *et al.,* 2006) until xylem flow into the berry is reduced during early ripening (Creasy and Lombard 1993). The

delayed entry into the ripening phase and associated retardation of berry expansion of NAA-treated fruit (Figure 14d-f) might therefore explain the slower decline in calcium concentrations in NAA-treated grapes compared with Control grapes at the first two post-veraison sampling time points (Figure 17a). NAA-treated Shiraz grapes displayed a similar trend to increased calcium concentrations, but due to large within-treatment variations, there were no significant differences in concentration between NAA-treated and Control fruit (data not shown). As calcium is known to stabilise cell walls through interaction with demethylated pectins (Demarty *et al.,* 1984) it is possible that the differences observed in Ca concentration in this study (Figure 17a) may influence cell walls in NAA-treated fruit.

Unlike calcium, potassium is mainly translocated in the phloem and its accumulation in grapes is highly correlated with berry sugar content and growth (Rogiers *et al.,* 2006). It is thought that potassium is essential for cell expansion by maintaining proton-pump activity (Stiles and van Volkenburgh 2004) and by contributing to the maintenance of an osmotic potential gradient between source leaves and berries, thereby enabling sugar import into ripening fruit (Véry and Sentenac 2003). Neither differences in sugar accumulation rate nor growth (Figure 14d,e) can explain the reduced potassium concentrations in the later ripening stages of NAA-treated grapes compared with Control fruit (Figure 17b) and there was no indication of a similar difference in accumulation patterns in Shiraz (data not shown).

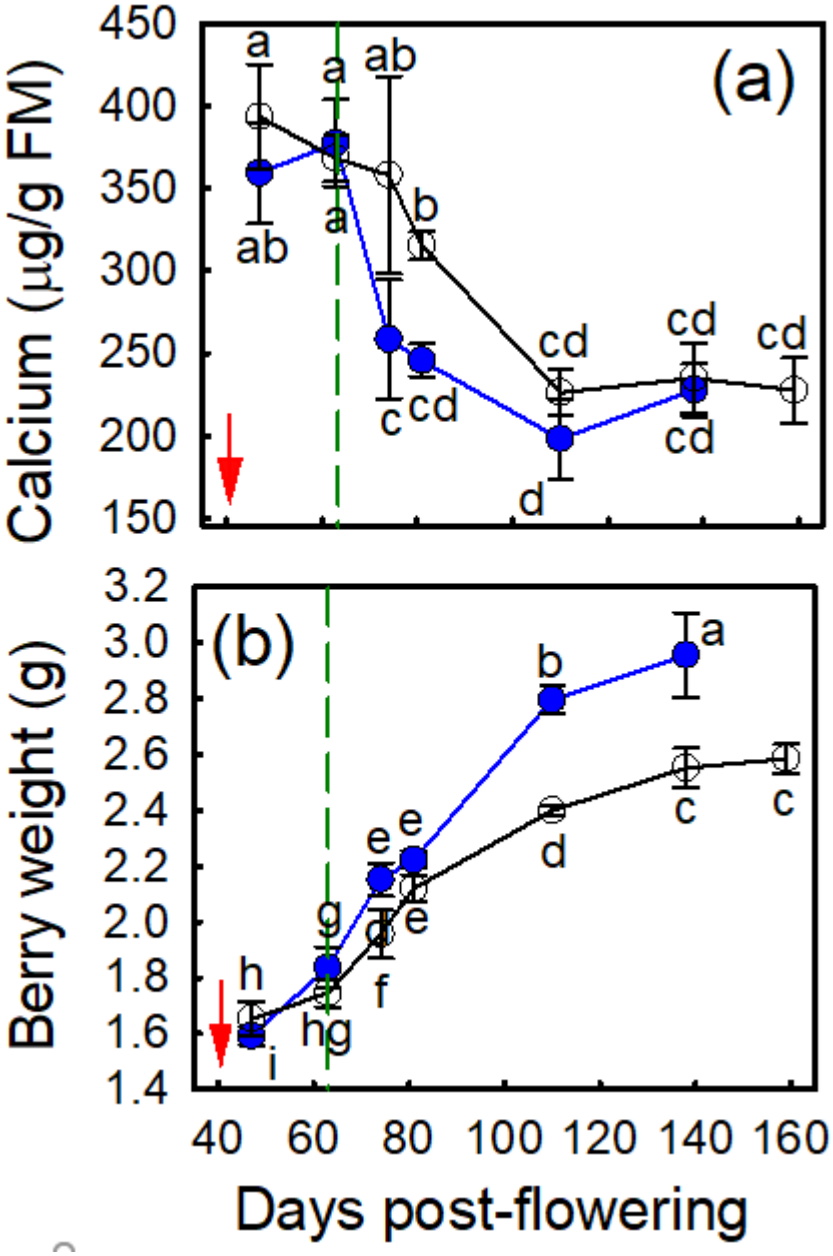


Figure 17. Calcium (a) and potassium (b) accumulation during development of Cabernet Sauvignon berries treated with Control solution (blue symbol and line) or with NAA (open circle, black line). Red arrows indicate time of (initial) spray and green, dashed lines mark veraison of the Control fruit. Values represent means ± SE (n = 3) and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test.

Grapes were also analysed for two compounds important to the distinctive varietal character of the two cultivars used in this study – rotundone for Shiraz and 3-isobutyl-2-methoxypyrazine (IBMP) for Cabernet Sauvignon (Figure 18). Both compounds are synthesised by the fruit and transfer directly into wine where they contribute important flavour and aroma attributes (Siebert *et al.,* 2008; Ryona *et al.,* 2009). The sesquiterpene rotundone confers a distinctive pepper character to Shiraz grapes and wine, which is considered an important character in cool climate Australian Shiraz (Wood *et al.,* 2008). In agreement with a previous study, where ripening delay by NAA resulted in increased concentrations of rotundone in

Shiraz wines (Davies *et al.,* 2015), NAA-treated Shiraz grapes contained a two-fold higher concentration of rotundone at the harvest date of the Control fruit, which increased to 37 ng/L by the time the NAA fruit was harvested (Figure 18a). This amounted to a 3.6-fold higher rotundone content in NAA grapes compared with Control grapes at the same harvest TSS and confirms the potential for NAA applications as a management tool to enhance the pepper character of Shiraz wines. Possible causes for the increased rotundone content include a direct effect of auxin on rotundone metabolism and possibly sesquiterpene metabolism in general (Davies *et al.,* 2015), the increased accumulation of the rotundone precursor α-guaiene in pre-veraison grapes due to the extension of this period and the longer hang time during the later stages of ripening, allowing for more rotundone to be produced by oxidation of α-guaiene (Huang *et al.,* 2014).

Methoxypyrazines are major contributors to the typicity and consumer preference of Cabernet Sauvignon wines, imparting herbaceous and green sensory attributes (Bowers and Meredith 1997). Methoxypyrazine concentrations in Cabernet Sauvignon grapes rapidly decrease during the post-veraison phase (Dunlevy *et al.,* 2010, 2013) and are negatively correlated with time-on-vine (Sala *et al.,* 2005). To determine any effect of NAA-induced ripening delay on the grape content of the most abundant methoxypyrazine, IBMP (Allen *et al.,* 1994), was measured (Figure 18b). NAA treatment did not cause any changes in IBMP concentrations during berry development. The lack of effect of NAA on IBMP concentration is another confirmation that NAA is unlikely to affect the concentration of compounds contributing to key sensory attributes, sesquiterpenes in Shiraz being the exception.

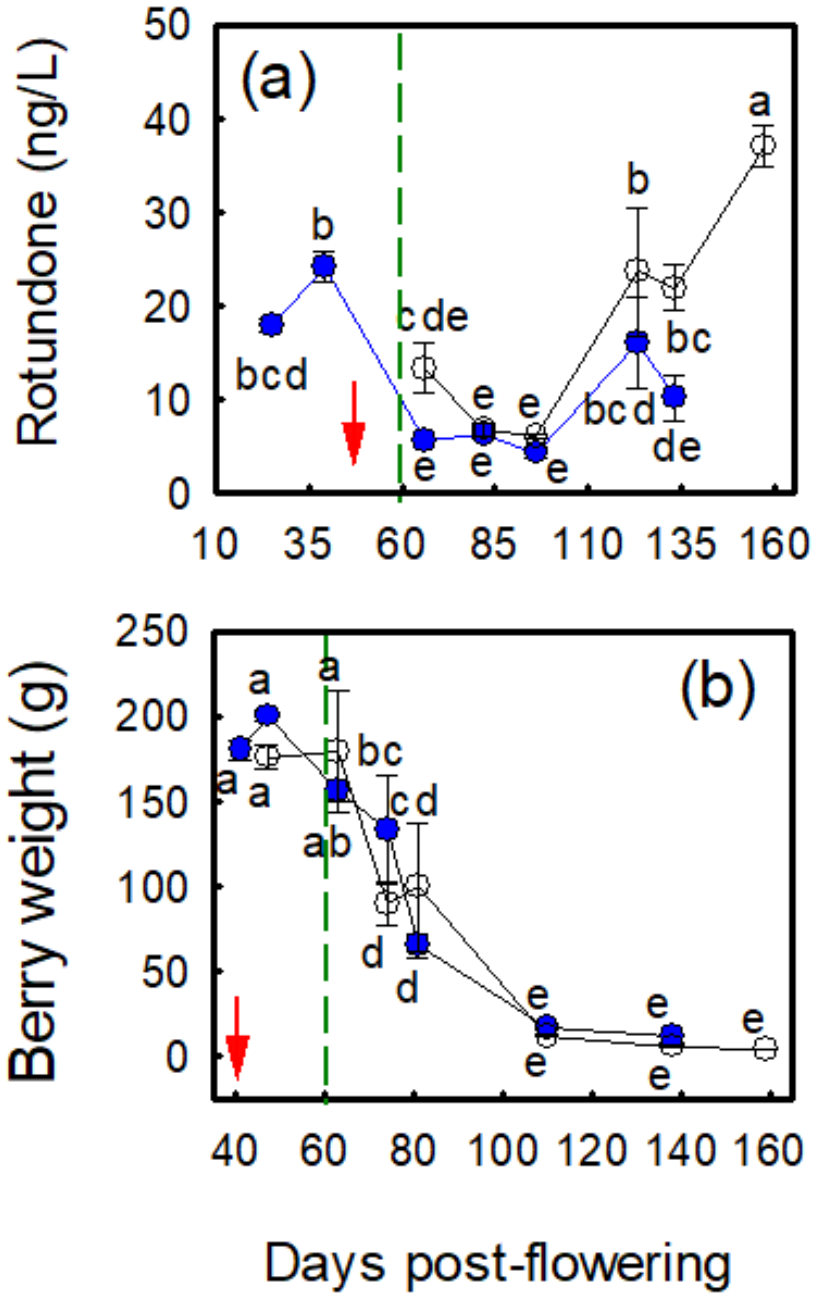


Figure 18. Rotundone accumulation during development of Shiraz berries (a) and 3-isobutyl 1-2-methoxypyrazine (IBMP) accumulation in developing Cabernet Sauvignon berries (b) treated with Control solution (blue symbol and line) or with NAA (open circle, black line). Red arrows indicate time of (initial) spray and green, dashed lines mark veraison of the Control fruit. Values represent means ± SE (n = 3) and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test.

Overall, it can be concluded that pre-veraison NAA-treatments delay berry development, promote cell wall stability and induce post-veraison rotundone accumulation, but otherwise have little effect on the basic biochemical properties of winegrapes that have been assessed to this point.

6.2.3.2 Auxin-induced changes to wine composition

Wines were made from Control and NAA-treated fruit to assess if NAA-induced ripening delay caused any changes to the broader range of compounds involved in contributing to wine sensory properties. At bottling (Table 8), there were no significant differences in alcohol content, pH, titratable acidity, sugar content and specific gravity between Shiraz Control and NAA wines, whereas small, but statistically significant, reductions in alcohol content, titratable acidity and specific gravity were detected in Cabernet Sauvignon NAA wines compared with Control wines. Since the average ethanol difference threshold in red wine is reported to be about 1% v/v (Yu and Pickering 2008), the observed difference in ethanol concentration is unlikely to affect wine volatile composition or sensory properties.

Table 8. Basic properties of wines made from Control and NAA-treated Shiraz (S) and Cabernet Sauvignon (CS) fruit at bottling.

Cultivar	Treatment	Bottling data				
		Ethanol (% v/v)	pH	TA 8.2 (g/L)	Glc+Fru (g/L)	SG (g/L)
S	Control	14.83	3.65	6.30	0.50	0.9918
	NAA	14.47	3.66	5.73	0.60	0.9912
CS	Control	15.00	3.65	6.30	0.53	0.9925
	NAA	14.27*	3.64	5.80*	0.50	0.9916*

Values are the mean of n = 3 biological replicates. * indicates significant difference between Control and NAA wines (t-test, $P < 0.05$) Fru, fructose; Glc, glucose; SG, specific gravity; TA, titratable acidity.

With the exception of rotundone (Davies *et al.,* 2015; Figure 18a), changes to the volatile profile of Shiraz and Riesling wines made from NAA-treated grapes have previously been reported to be minor and no consistent changes to the concentration of particular volatile compound(s) have been identified (Böttcher *et al.,* 2011b, 2012a; Davies *et al.,* 2015). It therefore seems that vintage, vineyard location and management and possibly treatment timing have a larger influence on the volatile composition of the wines than the NAA treatment of pre-veraison grapes itself. The general trend to small, but significant, changes in the concentration of some headspace volatile compounds in wines made from NAA-treated fruit was also confirmed in this study (Tables 9,10).

Table 9. Volatile compounds found to be significantly different (t-test, $P < 0.05$) in the headspace of wines produced from Shiraz berries that were treated with a Control solution or NAA.

Compound	Compound class	Control[a]	NAA[a]	Fold-change	LRI[b]	ID[c]
Higher abundance in Control wines						
1-Propanol	Alcohol	131.63	89.27	1.47	1044	A
1-Penten-3-ol	Alcohol	26.94	14.27	1.89	1158	A
3-Methyl-1-butanol[d]	Alcohol	14593.12	12817.91	1.14	1203	A
1-Pentanol[d]	Alcohol	22.89	19.06	1.20	1234	A
4-Methyl-pentanol[d]	Alcohol	116.57	73.45	1.59	1294	A
3-Methyl-pentanol[d]	Alcohol	324.28	215.46	1.51	1306	A
1-Hexanol[d]	Alcohol	6515.05	4474.76	1.46	1334	A
(*E*)-3-Hexen-1-ol	Alcohol	22.81	15.47	1.47	1343	A
(*Z*)-3-Hexen-1-ol	Alcohol	179.10	100.17	1.79	1362	A
1-Decanol[d]	Alcohol	8.42	6.40	1.32	1746	A
Ethyl 2-methylpropanoate	Ester	0.32	0.15	2.15	967	A
Ethyl 2-methylbutanoate	Ester	0.46	0.21	2.20	1055	A
Ethyl 3-methylbutanoate	Ester	0.62	0.28	2.21	1073	A
3-Methylbutyl propionate	Ester	0.25	0.11	2.37	1177	A
3-Methylbutyl 2-methylpropanoate	Ester	0.04	0.01	2.93	1182	B
3-Methylbutyl butanoate	Ester	0.13	0.07	1.83	1248	A
2-Methylpropyl hexanoate	Ester	0.05	0.03	2.00	1336	B
3-Methylbutyl hexanoate	Ester	0.41	0.24	1.90	1443	A
Propyl octanoate	Ester	0.02	0.01	1.86	1502	A
Ethyl 3-nonenoate[d]	Ester	1.79	1.17	1.54	1571	C
Ethyl salicylate[d]	Ester	0.05	0.02	2.07	1794	B
Ethyl hexanoate	Ester	3.82	2.06	1.86	1216	A
Ethyl octanoate[d]	Ester	2.79	1.47	1.90	1415	A
Ethyl decanoate	Ester	0.48	0.24	2.03	1624	A
p-Cymene	Monoterpene	7.80	6.14	1.27	1249	A
β-Citronellol	Monoterpene	182.61	122.01	1.50	1749	A
Theaspirane A	Norisoprenoid	6.09	1.89	3.22	1480	B
β-Cyclocitral[d]	Norisoprenoid	4.75	2.53	1.88	1600	B
β-Ionone[d]	Norisoprenoid	7.57	3.12	2.42	1923	A
n-Decanoic acid	Acid	159.55	99.78	1.60	>2000	A
2-Undecanone[d]	Ketone	0.53	0.21	2.57	1581	A
Styrene	Benzene	30.33	16.71	1.82	1237	A
Higher abundance in NAA wines						
1-Octen-3-ol[e]	Alcohol	17.46	26.99	1.55	1429	A
1-Octanol	Alcohol	145.29	194.10	1.34	1536	A
Benzaldehyde[e]	Aldehyde	22.81	107.30	4.70	1503	A
4-Terpineol[d]	Monoterpene	0.13	0.28	2.08	1581	A
δ-Cadinene	Sesquiterpene	0.31	0.51	1.66	1742	B

[a]Area under the peak of the selected ion relative to the relevant internal standard. Values are the mean of $n = 3$ biological replicates, [b]calculated from retention relative to the retention of a series of n-alkanes (C8-C26). [c]A=identity confirmed by matching weight spectra and LRI with that of authentic standards; B=tentative assignment based upon comparison with NIST05 and Wiley Registry (7th edition) weight spectral libraries, and published retention times; C=tentative identification of compound class suggested by comparison of weight spectra with those of related compounds in compound libraries. [d]Higher abundance in Cabernet Sauvignon Control wines (Table 10). [e]Higher abundance in Cabernet Sauvignon NAA wines (Table 10). ID, codes for identification; LRI, linear retention index

In Shiraz wines, 37 of 189 compounds identified by SPME-GC/MS were significantly different in concentration in the headspace of wines made from Control fruit compared with wines made from NAA-treated grapes, however most of them had a less than two-fold difference between the wines (Table 9). Higher alcohols and esters made up 70% of the wine volatiles with significant difference and the abundance of 32 of the 37 affected compounds (86%) was higher in Control wines than NAA wines.

In Cabernet Sauvignon wines, 57 of 155 identified compounds displayed significantly different headspace concentrations, with 52 volatiles at higher concentrations in Control wines and five volatiles at higher concentrations in NAA wines (Table 10). Similar to Shiraz, alcohols and esters made up the main classes of affected volatile compounds, but there was also a high number of terpenes (13) with higher concentration in Cabernet Sauvignon Control wines. As described for Shiraz, fold-change differences between Control and NAA wines were mostly small.

Some of the volatile compound differences between Control and NAA wines were observed for both cultivars analysed in this study and might therefore reflect generic responses to the NAA treatment rather than genetic differences or a range of possible vineyard management and weather events that occurred after harvest of the Control fruit. Thirteen of the volatile compounds with increased abundance in Shiraz Control wines were also found to be elevated in Cabernet Sauvignon Control wines (Tables 9,10). Of these, five alcohols (3-methyl-1-butanol, 3-methyl-pentanol, 4-methyl-pentanol, 1-hexanol, 1-decanol), have previously been identified as increased in Control wines of Shiraz and/or Riesling when compared with NAA wines (Böttcher *et al.,* 2011b; Böttcher *et al.,* 2012a; Davies *et al.,* 2015). This may suggest that some NAA-induced changes to amino acid and lipid metabolism of grapes and/or fermenting yeast are robust and independent of cultivar, vintage, vineyard location and even application timing and rate.

Despite few commonalities between NAA-affected wine volatile profiles across the studies available, it seems that NAA treatment might reduce lipoxygenase-derived lipid oxidation in ripening grapes. This is shown by the decreased concentration of C_6 and C_9 alcohols most likely derived from the lipoxygenase pathway (1-hexanol, 2-ethyl-hexanol, (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, 1-nonanol, (*Z*)-6-nonen-1-ol; Frankel 1983) in NAA wines from one or both of the cultivars used in this study (Tables 9,10) and in Shiraz and Riesling wines made from NAA-treated berries in previous experiments (Böttcher *et al.,* 2011b; Böttcher *et al.,* 2012a; Davies *et al.,* 2015).

Two volatile compounds, benzaldehyde and 1-octen-3-ol, were found to have increased concentrations in NAA wines of both cultivars and with a 4.7-fold (Cabernet Sauvignon) and 10.2-fold (Shiraz) increase, benzaldehyde was the compound with the biggest difference between Control and NAA wines. Neither compound has previously been associated with the NAA treatment of grapes, but both compounds have recently been linked to bunch rot of Chardonnay grapes (Steel *et al.,* 2020). As NAA-treated grapes were left for an additional 21 (Cabernet Sauvignon) and 24 days (Shiraz) on the vines after harvest of the Control fruit, during which time no further fungicide treatments occurred, it is plausible that a low level of grey mould infection was present in the NAA-treated grapes at harvest. 1-octen-3-ol is considered to provide a negative 'mushroomy' character to wine. However, whatever the reason, the concentration of 1-octen-3-ol was still low in the NAA-treated grapes.

Table 10. Volatile compounds found to be significantly different (t-test, $P < 0.05$) in the headspace of wines produced from Cabernet Sauvignon berries that were treated with a Control solution or NAA.

Compound	Compound class	Control[a]	NAA[a]	Fold-change	LRI[b]	ID[c]
Higher abundance in Control wines						
1-Butanol	Alcohol	188.90	109.83	1.72	1154	A
3-Methyl-1-butanol[d]	Alcohol	15802.21	12602.56	1.25	1207	A
1-Pentanol[d]	Alcohol	18.51	12.09	1.53	1237	A
3-Methyl-3-buten-1-ol	Alcohol	3.02	2.12	1.43	1237	A
4-Methyl-pentanol[d]	Alcohol	90.89	57.32	1.59	1296	A
3-Methyl-pentanol[d]	Alcohol	285.77	162.19	1.76	1307	A
1-Hexanol[d]	Alcohol	2256.69	1422.86	1.59	1334	A
3-Ethoxy-1-propanol	Alcohol	98.82	54.63	1.81	1356	A
2-Octanol	Alcohol	3.43	2.15	1.60	1399	B
1-Heptanol	Alcohol	25.76	15.26	1.69	1434	A
2-Ethyl-hexanol	Alcohol	127.55	38.52	3.31	1442	A
1-Nonanol	Alcohol	105.34	59.56	1.77	1641	A
(*Z*)-6-Nonen-1-ol	Alcohol	41.30	19.55	2.11	1666	A
3-Methylthio-1-propanol	Alcohol	216.21	132.78	1.63	1699	A
1-Decanol[d]	Alcohol	9.83	5.30	1.85	1746	A
Benzyl alcohol	Alcohol	180.24	110.47	1.63	1863	A
Phenol	Alcohol	6.95	5.82	1.19	2000	A
2-Phenylethanol	Alcohol	222.87	146.29	1.52	1898	A
2-Furancarboxaldehyde	Aldehyde	45.34	28.10	1.61	1445	A
2-Methyl-butanoate	Ester	466.28	314.48	1.48	1055	B
Ethyl 3-nonenoate[d]	Ester	5.84	2.38	2.46	1571	C
Diethyl pentanedioate	Ester	0.09	0.04	2.19	1762	A
Ethylphenyl acetate	Ester	3.91	2.16	1.81	1769	A
Ethyl salicylate[d]	Ester	0.04	0.02	1.97	1794	B
Geranyl acetate	Ester	0.06	0.04	1.55	1840	A
Phenylethyl butanoate	Ester	0.14	0.05	2.67	1949	A
Ethyl 5-oxooxolane-2-carboxylate	Ester	26.84	11.77	2.28	>2000	C
Ethyl hexanoate[d]	Ester	6.29	3.73	1.69	1217	A
Ethyl octanoate[d]	Ester	3.64	2.26	1.61	1415	A
α-Terpinene	Monoterpene	0.25	0.15	1.68	1155	A
m-Cymene	Monoterpene	24.99	17.35	1.44	1243	A
Linalool	Monoterpene	0.59	0.41	1.44	1528	A
α-Fenchol	Monoterpene	11.09	6.38	1.74	1564	B
4-Terpineol[e]	Monoterpene	0.23	0.17	1.37	1582	A
Hotrienol	Monoterpene	0.13	0.08	1.72	1591	B
α-Terpineol	Monoterpene	1.12	0.64	1.74	1678	A
Borneol	Monoterpene	12.14	6.74	1.80	1683	B
Nerol	Monoterpene	0.12	0.08	1.59	1783	A
trans-*β*-Farnesene	Sesquiterpene	7.86	3.77	2.09	1652	A
trans-Calamenene	Sesquiterpene	2.25	1.52	1.48	1817	B
Nerolidol	Sesquiterpene	14.65	6.20	2.36	>2000	A
Cadalene	Sesquiterpene	2.80	1.98	1.41	>2000	B
β-Cyclocitral[d]	Norisoprenoid	5.41	2.83	1.91	1599	B
β-Ionone[d]	Norisoprenoid	6.82	3.00	2.27	1923	A
Hexanoic acid	Acid	1573.50	1255.72	1.25	1850	A
Octanoic acid	Acid	1910.15	1499.21	1.27	>2000	A
1-(Furan-2-yl)ethanone	Ketone	3.61	2.17	1.66	1485	B
2-Undecanone[d]	Ketone	0.58	0.14	4.10	1581	A
Compound	**Compound class**	**Control[a]**	**NAA[a]**	**Fold-change**	**LRI[b]**	**ID[c]**
γ-Butyrolactone	Lactone	101.59	50.37	2.02	1613	A
4,5-Dimethyl-1,3-benzenediol	Benzene	13.27	9.17	1.45	1613	C
1,2-Dihydro-4,5,7-trimethylnaphthalene	Hydrocarbon	1.86	1.11	1.67	1811	C
Higher abundance in NAA wines						
1-Octen-3-ol[e]	Alcohol	19.19	34.69	1.81	1430	A
Benzaldehyde[e]	Aldehyde	23.03	234.00	10.16	1502	A

Acetic acid	Acid	386.78	577.79	1.49	1457	A
Butanoic acid	Acid	36.56	42.57	1.16	1635	A
3-Octanone	Ketone	3.15	5.86	1.86	1233	A

[a]Area under the peak of the selected ion relative to the relevant internal standard. Values are the mean of *n* = 3 biological replicates, [b]calculated from retention relative to the retention of a series of n-alkanes (C8-C26). [c]A=identity confirmed by matching weight spectra and LRI with that of authentic standards; B=tentative assignment based upon comparison with NIST05 and Wiley Registry (7th edition) weight spectral libraries, and published retention times; C=tentative identification of compound class suggested by comparison of weight spectra with those of related compounds in compound libraries. [d]Higher abundance in Shiraz Control wines (Table 9). [e]Higher abundance in Shiraz NAA wines (Table 9). ID, codes for identification; LRI, linear retention index

6.2.3.3 RATA sensory evaluation of wines made from Control and auxin-delayed fruit

Previous studies investigating the effect of NAA-induced ripening delay on the sensory properties of Shiraz and Riesling wine reported either no (Böttcher *et al.,* 2011b) or few (Böttcher *et al.,* 2012a; Davies *et al.,* 2015) sensorial differences between the wines made from NAA-treated and Control fruit. Given the long ripening delay of 21 (Cabernet Sauvignon) and 24 (Shiraz) days achieved in the current study and the lack of information about the effect of NAA treatment on Cabernet Sauvignon wines, a RATA sensory panel (n = 60) was used to expand the existing knowledge about the influence of ripening delay by pre-veraison NAA application on wine sensory characteristics and to allow a comparison between the two cultivars.

The RATA assessment of the Shiraz wines identified ten of the 47 sensory attributes as significantly different ($P < 0.05$) between the wines made from NAA-treated and Control fruit and Principal Component Analysis (PCA) was used to produce a biplot of those attributes, the wines, and selected wine/grape compounds (Figure 19).

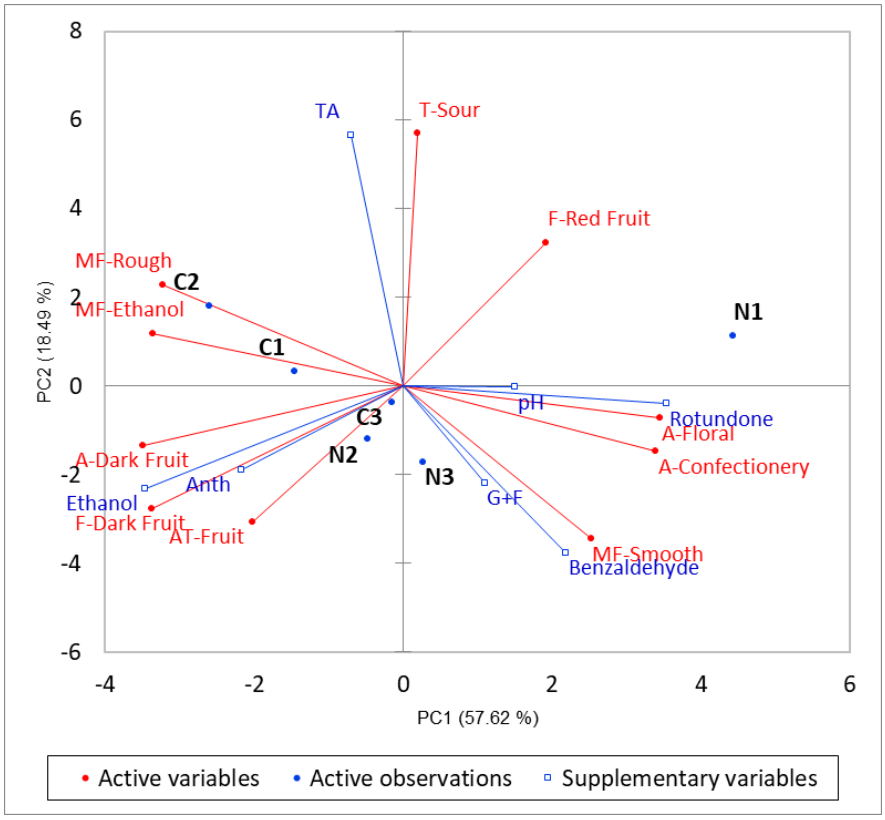


Figure 19. PCA biplot of mean RATA scores for significantly different sensory attributes (red) overlaid with chemical data (blue) for Control (C1-C3) and NAA (N1-N3) Shiraz wines (black). A-, aroma, Anth, grape anthocyanins, AT-, aftertaste, F-, flavour, Fru, fructose, Glc, glucose, MF-, mouthfeel, T-, taste, TA, titratable acidity.

The PCA of the mean RATA scores for significantly different sensory attributes explained 76.11% of the data variation with the first two principal components (PC1 = 57.62%, PC2 = 18.49%). PC1 was separated by red fruit flavour, floral and confectionery aromas, and smooth mouthfeel on the positive loadings side and dark fruit flavour and aroma, alcoholic and rough mouthfeel and a fruity aftertaste on the negative loadings side of the biplot. PC2 was primarily driven by sour taste with some red fruit influence opposed to confectionary notes and dark fruit attributes. While within-treatment variability led to a significant

spread of the replicate wines across the biplot, two out of the three NAA wines were associated with the positive PC1 loadings attributes and all three Control wines were linked to the negative PC1 loadings attributes. The dark fruit notes perceived in the Control wines and N2 aligned with increased anthocyanin content in the corresponding fruit (Figure 19), which confirms previous findings of a positive correlation between anthocyanins and dark fruit flavour (Niimi *et al.,* 2013). The additional discrimination of these four wines on PC1 by ethanol mouthfeel was supported by the ethanol content analysis of the wines (Figure 19). The chemical vector for titratable acidity and the sensory vector for sour taste were closely aligned in PC2, reinforcing the panel's accurate discrimination of wines based on acidity. The increased concentration of benzaldehyde in the wines made from ripening delayed fruit (Table 10) might have influenced the perception of confectionary aroma linked to N1 and N3 wines (Figure 19) as this volatile compound is known to impart almond or marzipan notes to wines. Rotundone might also have contributed to wine discrimination as like the grapes (Figure 18a), NAA wines contained approximately 4.5-times higher rotundone concentrations (13.30 ng/L) than Control wines (2.98 ng/L). In accordance with the odour threshold limit of 16 ng/L (Wood *et al.,* 2008), the elevated rotundone levels in NAA wines did not translate into significant changes to the rating of pepper flavour and aroma by the RATA panel. However, it is feasible that the sub-threshold levels of this sesquiterpene compound may have intensified the perception of floral aroma notes or suppressed the perception of dark fruit flavours and aromas (Ryan *et al.,* 2008; Figure 19). It is also possible that the increase in rotundone accumulation in NAA wines was indicative of a more general induction of sesquiterpene biosynthesis (Davies *et al.,* 2015) or terpene production, which may have resulted in elevated floral aroma perception in N1 and N3 wines (Figure 19). It should also be remembered that some people can perceive rotundone below the threshold limit.

For the Cabernet and Control wines none of the 47 attributes evaluated by the panel were perceived to be significantly different (P = 0.05), confirming the above mentioned reports from other cultivars and further highlighting the potential of NAA as a tool to significantly delay berry ripening without impacting the sensorial perception of the resulting wine.

6.2.4 Conclusions

This study has demonstrated that pre-veraison applications of NAA can be used to delay the ripening and harvest of Shiraz and Cabernet Sauvignon grapes by 3-4 weeks without causing any significant changes to basic fruit and wine parameters typically assessed for winemaking purposes. Modifications in nutrient accumulation were minor as were the differences in the basic properties of wines made from NAA and Control fruit. A positive side effect of the auxin treatments was the observed maintenance of berry integrity, possible related to structural cell wall changes. The combined results of wine volatile compound analysis and sensorial assessment by RATA suggest that the overall effect of NAA-induced ripening delay on the flavour and aroma composition of wine is small, but that cultivar-specific variations may be observed. This was illustrated by increased concentrations of the pepper aroma compound rotundone in NAA-treated Shiraz fruit and corresponding wine, which might have contributed to the sensorial distinction of Shiraz NAA and Control wines, whereas, in line with the lack of change in IBMP, no significant sensorial differences were detected in the equivalent Cabernet Sauvignon wines. Overall, these findings promote the use of NAA as a viticultural management tool to mitigate the adverse effects of accelerated ripening and harvest season compression on wine properties and harvesting and wine making logistics. NAA could be used commercially as a ripening-delay agent to delay grape maturation and de-compress harvest whilst preserving berry integrity and wine typicity.

6.3 Determination of the effect of auxin application timing on its effectiveness as a delayer of ripening

6.3.1 Introduction

The timing of NAA application will almost certainly be critical to the length of delay in ripening/harvest and needs to be carefully investigated. Application too long before veraison could result in auxin being metabolised too early before veraison thus reducing the concentration of active NAA and hence there may be little or no delay. An application after the commencement of veraison does not elicit a delay in ripening or harvest (data not shown), as once committed to ripening, auxin application has no delaying effect. Accurate timing will allow more targeted application, reducing 1) the need for higher application rates of NAA, 2) the costs of reagents and 3) any residue levels, while maintaining efficacy. Even in the best-managed vineyards there is a degree of asynchronous development within and between bunches and between different vines and so an inappropriate timing of NAA application could result in a greater degree of asynchronous ripening. For these reasons a better understanding of the time window most suitable for application is required.

In this experiment we tested the effect the timing of NAA application has on the length of harvest delay, the effect on berry ripening synchronicity, berry size and berry and wine composition under commercial conditions.

6.3.2 Materials and methods

A field experiment to determine the effect of timing of NAA application on berry phenology and composition and wine character was conducted using Cabernet Sauvignon vines. These double cordon vines were located in a drip-irrigated, commercial vineyard at Padthaway, South Australia. The trial was of a randomised, triplicated design with four vines used for each replicate (Figure 20). Control bunches were sprayed with a solution of wetting agent only (0.1% (v/v) Vitiwet, SST Products, South Melbourne, Australia). Auxin-treated bunches were sprayed with the same solution but containing 50 mg/L NAA (Gibco BRL Life Technologies, Grand Island, USA). Treatments were carried out at three stages of berry development, treatment 1 (T1), 25 days pre-veraison (21 Dec), treatment 2 (T2), 16 days pre-veraison (30 Dec), treatment 3 (T3), at veraison (15 Jan). Veraison was determined as the sampling timepoint prior to the first observed increase in TSS. All of the bunches on four vines were used for each Control and NAA-treated replicate. A separate Control treatment was done for each NAA treatment. On three dates, 60 berries from each replicate were harvested, weighed and TSS measured as Brix using a digital refractometer. For the remainder of the experiment, samples of 100 berries were taken at various times (as shown in figures) for each replicate from each treatment, and weight and TSS measured for individual berries to assess the degree of variability. Berry weight and TSS data were processed to determine the level of synchronicity using all of the weight and Brix values for each treatment (approximately 300 berries for each treatment at each sampling time). The individual values for TSS and berry weight were then distributed into classes of 0.5 °Brix and 0.05 g respectively and these aggregated data were plotted as distribution curves. At harvest, approximately 200 g of berries were frozen immediately, transported on dry ice and stored at -80°C.

Row 1	Row 2	Row 3
C1	N3	C2
N1	C3	N2
C2	N1	C3
N2	C1	N3
C3	N2	C1
N3	C2	N1

Figure 20. Triplicated, randomised trial design for testing the relationship between the timing of NAA treatment and its efficacy. The three Control (orange shading) and NAA (black shading) replicates were spread across three rows of vines. C1 and N1 represent the earliest treatment time (25 days pre-veraison) followed by C2 and N2 (16 days pre-veraison) and C3 and N3 at veraison).

6.3.3 Results/Discussion

6.3.3.1 The effects of NAA treatment on berry weight and TSS accumulation

The application of 50 mg/L NAA to Cabernet Sauvignon berries at three different stages, two pre-veraison (N1, N2, treated 25 and 16 days pre-veraison, respectively) and one at veraison (N3) (Figure 20) altered berry weight when compared with the three respective Controls C1, C2 and C3 (treated on the same days as N1, N2, N3, Figure 21). The timing of the weight increase of N1 and N2 berries during the ripening phase was delayed compared with that of the Controls, but once they commenced expansion, they did so more rapidly than the Control berries. The N3 berries displayed a different pattern as the increase in berry weight was not delayed by the treatment and it closely followed the pattern of berry weight increase of the Controls until the Controls reached their maximal weight at 99 days post- flowering (dpf). The anomalous shape of the N3 berry weight curve can be explained by the different sampling times for Control and NAA-treated berries. Dates for sampling were chosen to ensure that the fruit from the various Control and NAA treatments were taken at similar TSS concentrations. Hence, the dates for sampling the NAA treatments were often different from those of the Controls and there were also differences between the sampling dates for the N1, N2, and N3 treatments (Figure 21, Table 11). Therefore, although the contour of berry weight increase in N3 berries appeared quite different to N1 and N2 this perception may have resulted from differences in sampling frequency and timing. This agrees with a much lower correlation between %SD for Brix at veraison and %SD for Brix at the 20 °Brix stage.

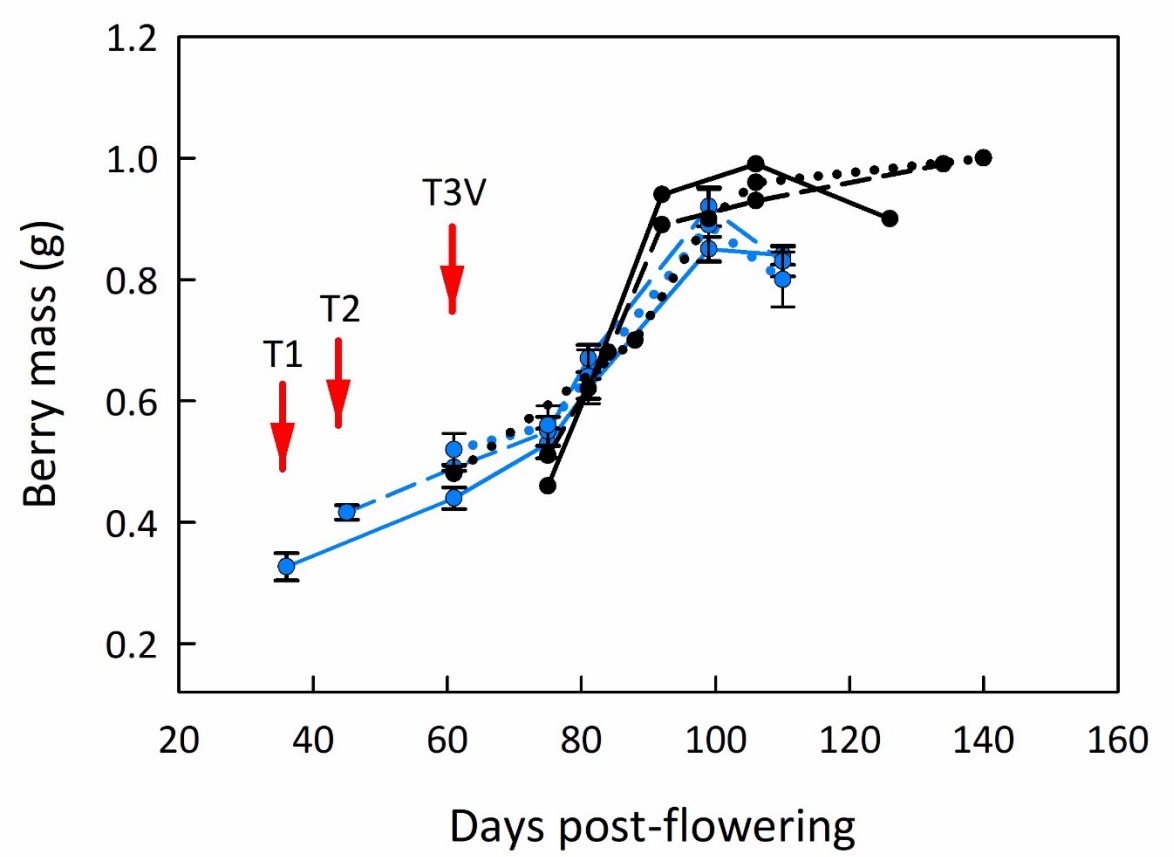


Figure 21. Weight of Control treatment 1 (blue solid line), Control treatment 2 (blue dashed line), Control treatment 3 (blue dotted line) and NAA treatment 1 (black solid line), NAA treatment 2 (black dashed line) and NAA treatment 3 (black dotted line) berries. Arrows indicate time of NAA treatments (T1, T2, T3V), the T3V label indicates that the time of veraison which was coincident with NAA T3. Statistical analysis is provided in Table 11.

Table 11. Statistics for Figure 21. ANOVA followed by Duncan's post hoc ($P < 0.05$). DPF = days post flowering.

Treatment	DPF	Berry weight	Duncan's post hoc
Control 1	36	0.33 ± 0.02	n
Control 2	45	0.42 ± 0.01	m
Control 1	61	0.44 ± 0.02	lm
Control 2	61	0.49 ± 0.01	klm
Control 3	61	0.52 ± 0.03	klm
NAA 3	61	0.48 ± 0.02	klm
Control 1	75	0.53 ± 0.02	kl
Control 2	75	0.55 ± 0.02	jk
Control 3	75	0.56 ± 0.03	Ijk
NAA 1	75	0.46 ± 0.02	lm
NAA 2	75	0.51 ± 0.01	kl
Control 1	81	0.62 ± 0.02	ghij
Control 2	81	0.67 ± 0.02	gh
Control 3	81	0.64 ± 0.04	ghi
NAA 1	81	0.62 ± 0.02	hij
NAA 2	84	0.68 ± 0.04	gh
NAA 3	88	0.70 ± 0.05	g
NAA 1	92	0.94 ± 0.01	ab
NAA 2	92	0.89 ± 0.03	bcdef
Control 1	99	0.85 ± 0.02	cdef
Control 2	99	0.92 ± 0.03	abcd
Control 3	99	0.89 ± 0.06	bcdef
NAA 3	99	0.90 ± 0.06	bcde
NAA 1	106	0.99 ± 0.01	a
NAA 2	106	0.93 ± 0.03	abc
NAA 3	106	0.96 ± 0.04	ab
Control 1	110	0.84 ± 0.02	def
Control 2	110	0.83 ± 0.02	ef
Control 3	110	0.80 ±0.05	f
NAA 1	126	0.90 ± 0.02	bcde
NAA 2	134	0.99 ± 0.08	a
NAA 3	140	1.00 ± 0.02	a

After reaching a maximum at 99 dpf for the Controls and at 106 dpf for N1, the average berry weight decreased, presumably due to dehydration, but the weight of the N2 and N3, NAA-treated berries remained at their maximum level until harvest. The phenomenon of NAA treatment resulting in higher berry harvest weight compared with Control fruit has been observed previously (Böttcher *et al.,* 2011b) but this experiment allows a more detailed assessment. The mean weight of N2 berries was 19% greater than C2 berries, and N3 berries weighed 25% more than C3 berries. This weight increase represents a significant yield increase for growers and winemakers that does not rely on increased flowering, pollination or fruitset, events, which are difficult to influence in the vineyard. The maintenance of berry weight in the NAA-treated fruit treated within 16 days before veraison indicates that these berries are resistant to late ripening-stage shrinkage. Although Shiraz is often cited as a cultivar particularly susceptible to berry shrivel disorder (Krasnow *et al.,* 2010), various degrees of shrivel have previously been reported in other cultivars including Cabernet Sauvignon (Krasnow *et al.,* 2009). Berry weight loss due to dehydration would cause the concentration of the hexoses contained in the berry to increase, causing an increase in TSS. This may be the reason for the higher rate of TSS accumulation late in the

ripening of Control fruit and conversely, the reason for the lower rate of TSS accumulation in NAA-treated berries at this stage (Figure 22, Table 12).

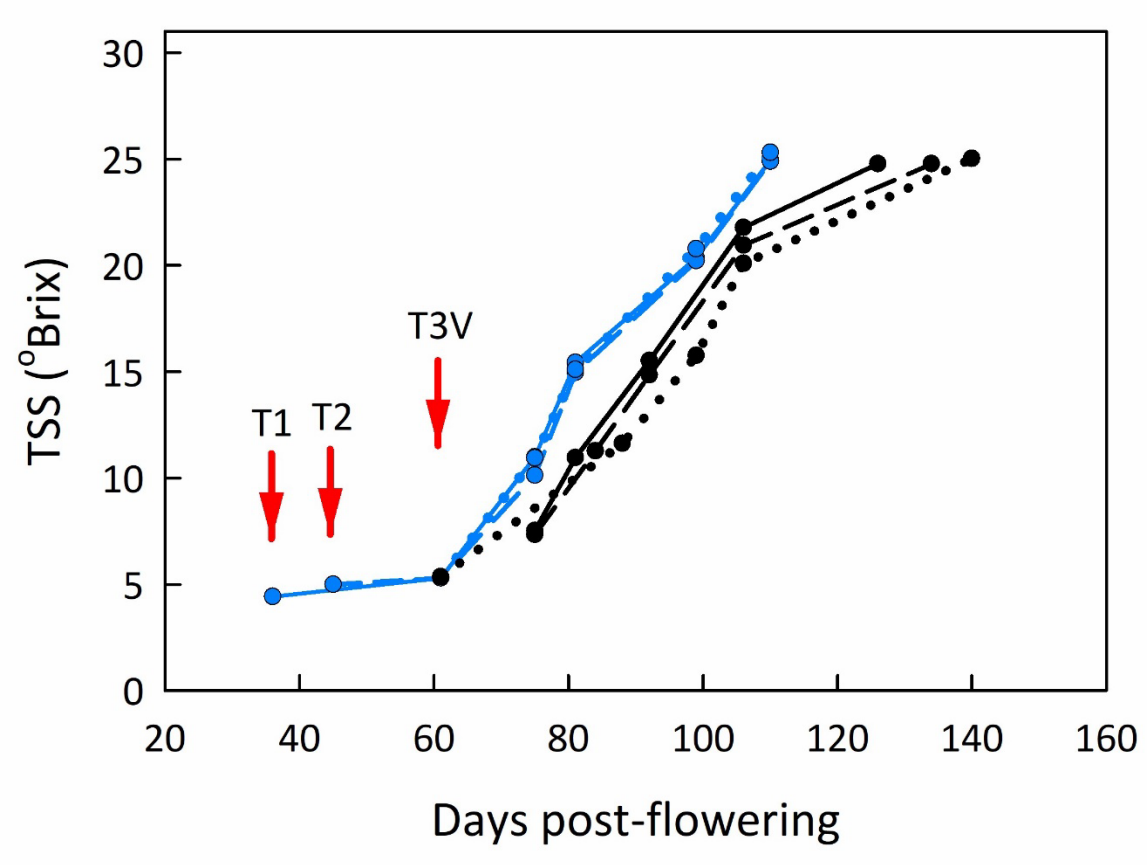


Figure 22. TSS accumulation (°Brix) of Control treatment 1 (blue solid line), Control treatment 2 (blue dashed line), Control treatment 3 (blue dotted line) and NAA treatment 1 (black solid line), NAA treatment 2 (black dashed line) and NAA treatment 3 (black dotted line) berries. Arrows indicate time of NAA treatments (T1, T2, T3V), the T3V label indicates that the time of veraison which was coincident with NAA T3. Statistical analysis is provided in Table 12.

Table 12. Statistics for Figure 22. ANOVA followed by Duncan's post hoc ($P < 0.05$). DPF= days post flowering.

Treatment	DPF	TSS (°Brix)	Duncan's post hoc
Control 1	36	4.43 ± 0.04	h
Control 2	45	5.00 ± 0.07	h
Control 1	61	5.29 ± 0.04	h
Control 2	61	5.30 ± 0.02	h
Control 3	61	5.30 ± 0.14	h
NAA 3	61	5.35 ± 0.11	h
Control 1	75	10.98 ± 0.42	ef
Control 2	75	10.13 ± 0.30	f
Control 3	75	10.94 ± 0.62	ef
NAA 1	75	7.53 ± 0.26	g
NAA 2	75	7.34 ± 0.35	g
Control 1	81	15.43 ± 0.29	d
Control 2	81	14.95 ± 0.22	d
Control 3	81	15.10 ± 0.31	d
NAA 1	81	10.95 ± 0.05	ef
NAA 2	84	11.27 ± 0.28	e
NAA 3	88	11.62 ± 0.43	e
NAA 1	92	15.52 ± 0.14	d
NAA 2	92	14.84 ± 0.21	d
Control 1	99	20.36 ± 0.72	c
Control 2	99	20.22 ± 0.32	c
Control 3	99	20.77 ± 0.19	c
NAA 3	99	15.76 ± 0.68	d
NAA 1	106	21.77 ± 0.15	b
NAA 2	106	20.95 ± 0.41	bc
NAA 3	106	20.09 ± 0.43	c
Control 1	110	24.95 ± 0.74	a
Control 2	110	24.89 ± 0.59	a
Control 3	110	25.31 ± 0.37	a
NAA 1	126	24.77 ± 0.18	a
NAA 2	134	24.77 ± 0.27	a
NAA 3	140	25.02 ± 0.39	a

There may be a number of causes of the maintenance of berry weight in the later stages of ripening of NAA-treated fruit. Considerable changes to the expression of many genes involved in a wide range of processes occur in auxin-treated grapes (Ziliotto *et al.,* 2012; He *et al.,* 2020, 2021; this report). Auxin-induced changes to the waxy cuticle might prevent berry weight loss through reduced transpiration. NAA-induced changes to cell wall components could have protected the treated fruit from undergoing weight loss by preserving cellular structure and membrane integrity. Changes to cell wall components and the expression of putative cell wall-associated genes have been observed in NAA treated berries (Dal Santo *et al.,* 2020). Cell death is thought to play a role in berry shrivel (Tilbrook and Tyerman 2008, Krasnow *et al.,* 2009; Hoff *et al.,* 2020) and NAA could delay or reduce the rate of cell death that is seen in berries that shrivel.

Measurements of TSS also showed that the timing of NAA application had a significant effect on sugar accumulation (Figure 22). The curves of TSS increase for the C1, C2 and C3 berries were not significantly different, with small standard errors, which shows that the berry sampling regime (berry number and sampling timing) was adequate for assessing changes in hexose accumulation. The NAA-treated berries showed a progressive increase in the length of the delay of TSS accumulation the later the treatment time

(Figure 22). The delay between the Control and the NAA-treated berries reaching approximately 25 °Brix at harvest was 16, 24, and 30 days for C1/N1, C2/N2, C3/N3, respectively i.e. the nearer the application was to veraison, the greater the delaying effect. Previous studies have shown that the application of auxins to grape berries induces a homeostatic response. One of several mechanisms involves an increase in the transcript level of enzymes that conjugate auxins, including NAA, to amino acids thus inactivating them (Böttcher *et al.,* 2010, 2011a). The application of NAA nearer to the time of veraison means that there is less time for the homeostatic responses to inactivate excess auxin, which could lead to a longer delay in ripening. The delay of the NAA-treated berries reaching 25 °Brix was in part due to a shift in veraison but also due to slower rates of TSS increase during the later stages of ripening. The higher rate of berry growth between 75 and 92 dpf of NAA-treated fruit contributed to slower TSS accumulation, as more sugars were required to 'fill' the larger berries (Figure 21). During the later stages of development of NAA-treated berries, 106 dpf to harvest, the rate of TSS accumulation was slower and this coincided with the maintenance of berry weight, particularly for N2 and N3 fruit (Figures 21, 22). The maintenance of berry weight and slower sugar accumulation could be an advantage for grapegrowers and winemakers, as it results in increased yield and longer 'hang times'.

The delay in veraison resulting from NAA treatment caused the later stages of ripening of the treated fruit to be shifted into a cooler part of the season (Figure 23). The mean maximum daily temperature for the period from the third NAA treatment (which was also veraison, TV3) until harvest of the Controls (CH) was 30.5°C, while that for the period from CH to the harvest of the third NAA-treated berries (N3H) was 24.5°C. This means that the later stages of ripening occurred at a lower temperature, which may have a positive effect on berry composition and the shift into cooler conditions may also further delay harvest. Fruit grown under cooler conditions usually demands a higher price, due to perceived improved quality.

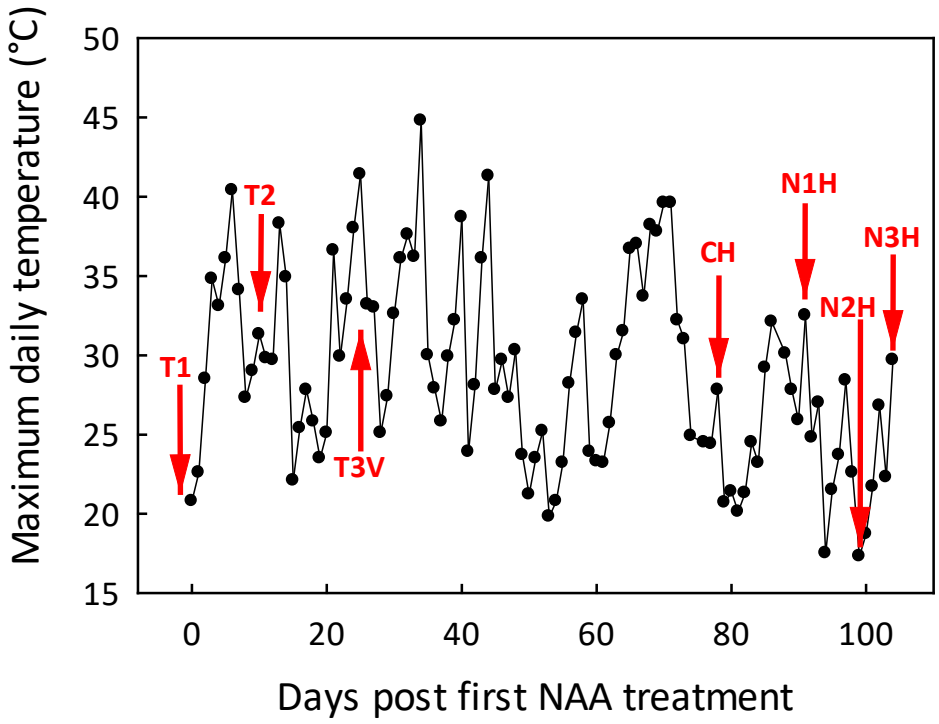


Figure 23. Maximum daily temperatures for the Padthaway vineyard site during the period from the first NAA treatment to the harvest of the third NAA treatment. T1 = NAA treatment 1, T2 = NAA treatment 2, T3V = NAA treatment 3 which coincided with veraison, CH = harvest of all three Control treatments, N1H, N2H and N3H = the harvest times of the three NAA treatments. Data obtained from The Australian Bureau of Meteorology (http://www.bom.gov.au.) weather station 26100 (Padthaway South).

6.3.3.2 NAA treatment increased net carbon accumulation

Net carbon accumulation can be estimated by the product of TSS level (°Brix) x berry weight (g). There was no significant difference in net carbon accumulation between the Controls. The differences in berry weight between Control and NAA-treated fruit, particularly for N2 and N3 meant that in the final stages of their development N2 and N3 berries stored significantly more carbon than the Controls or N1 (Figure 24, note that the statistics for this figure are contained in Table 13). The estimated increase in stored carbon compared to the respective Controls was 7% (N1), 19% (N2) and 24% (N3), which is consistent with the increase in berry weight (Figure 21). It therefore appears that the NAA-treated berries acted as a larger sink due to their increased size (as discussed above). As the measurements were taken from berries at the same mean TSS concentration, this represents a considerable increase in yield.

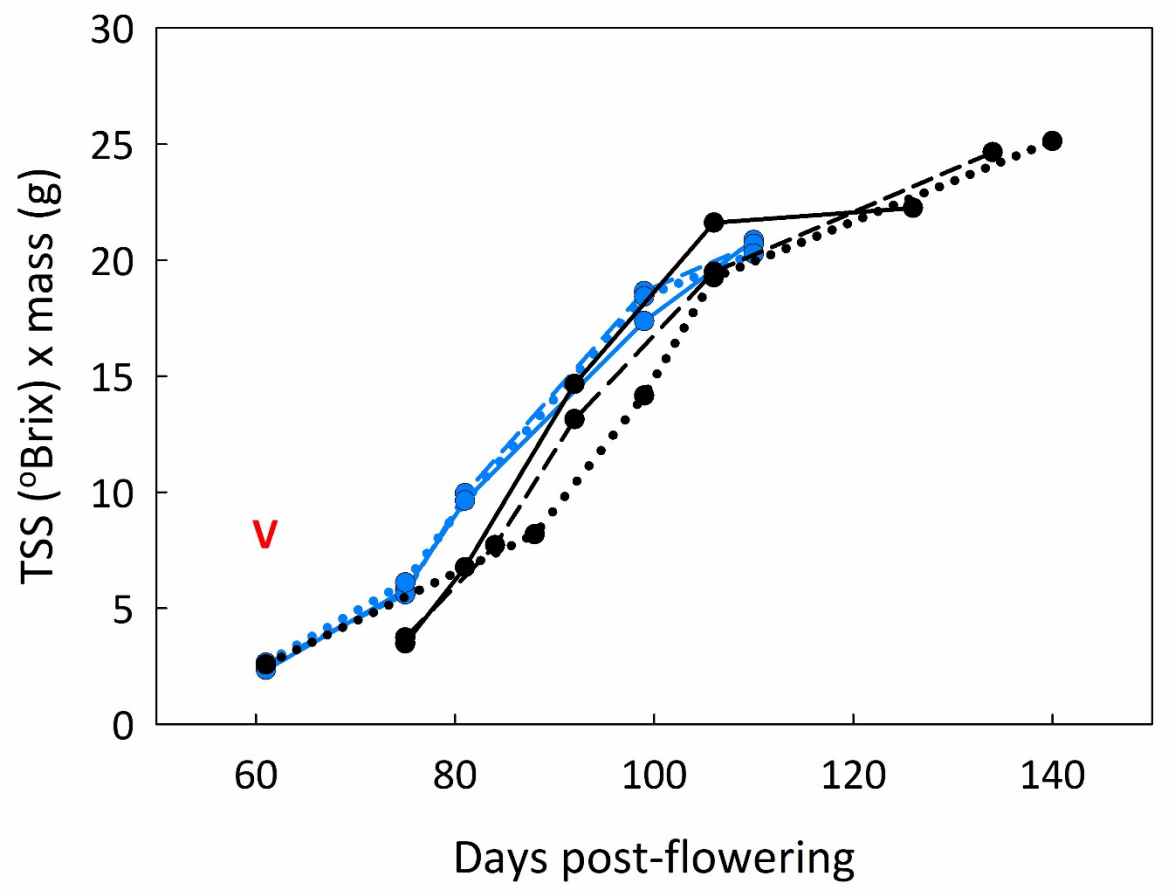


Figure 24. TSS x berry weight of Control treatment 1 (solid blue line), Control treatment 2 (dashed blue line), Control treatment 3 (dotted blue line) and NAA treatment 1 (solid black line), NAA treatment 2 (dashed black line) and NAA treatment 3 (dotted black line) berries. Veraison is indicated by V. Statistical analysis is provided in Table 12.

Table 13. ANOVA for Figure 24 followed by Duncan's post hoc ($P < 0.05$). DPF = days post flowering.

Treatment	DPF	TSS (°Brix)*weight	Duncan's post hoc
Control 1	61	2.34	l
Control 2	61	2.59	l
Control 3	61	2.64	l
NAA 3	61	2.57	l
Control 1	75	5.78	k
Control 2	75	5.59	k
Control 3	75	6.10	jk
NAA 1	75	3.47	l
NAA 2	75	3.73	l
Control 1	81	9.63	h
Control 2	81	9.95	h
Control 3	81	9.63	h
NAA 1	81	6.76	ljk
NAA 2	84	7.71	lj
NAA 3	88	8.18	hi
NAA 1	92	14.66	g
NAA 2	92	13.14	g
Control 1	99	17.37	f
Control 2	99	18.66	ef
Control 3	99	18.43	ef
NAA 3	99	14.16	g
NAA 1	106	21.62	bc
NAA 2	106	19.49	de
NAA 3	106	19.25	de
Control 1	110	20.86	bcd
Control 2	110	20.68	bcd
Control 3	110	20.28	cde
NAA 1	126	22.25	b
NAA 2	134	24.65	a
NAA 3	140	25.13	a

6.3.3.3 NAA treatment altered the synchronicity of berry weight and TSS

The distribution of berry weight for individual Control and NAA-treated berries, for the three different application times, was assessed at four developmental stages (Figure 25). The timing of sampling was chosen to provide samples with similar average TSS of 10, 15, 20 and 25 °Brix. Small differences occurred between these targeted mean values and the actual mean values (the actual values are provided in Figures 25, 27). At the 15 °Brix stage the NAA-treated berries were less synchronised than the Controls, having a broader weight distribution (Figure 25). This is further illustrated by significantly higher berry weight SD values for the N1, N2 and N3 berry populations compared with the Controls (Figure 26) at the 15 °Brix stage. At most stages, the distribution curves of the weight of the NAA-treated berries were skewed to the right of that of the Controls reflecting the significantly higher mean berry weight (Figures 21, 25).

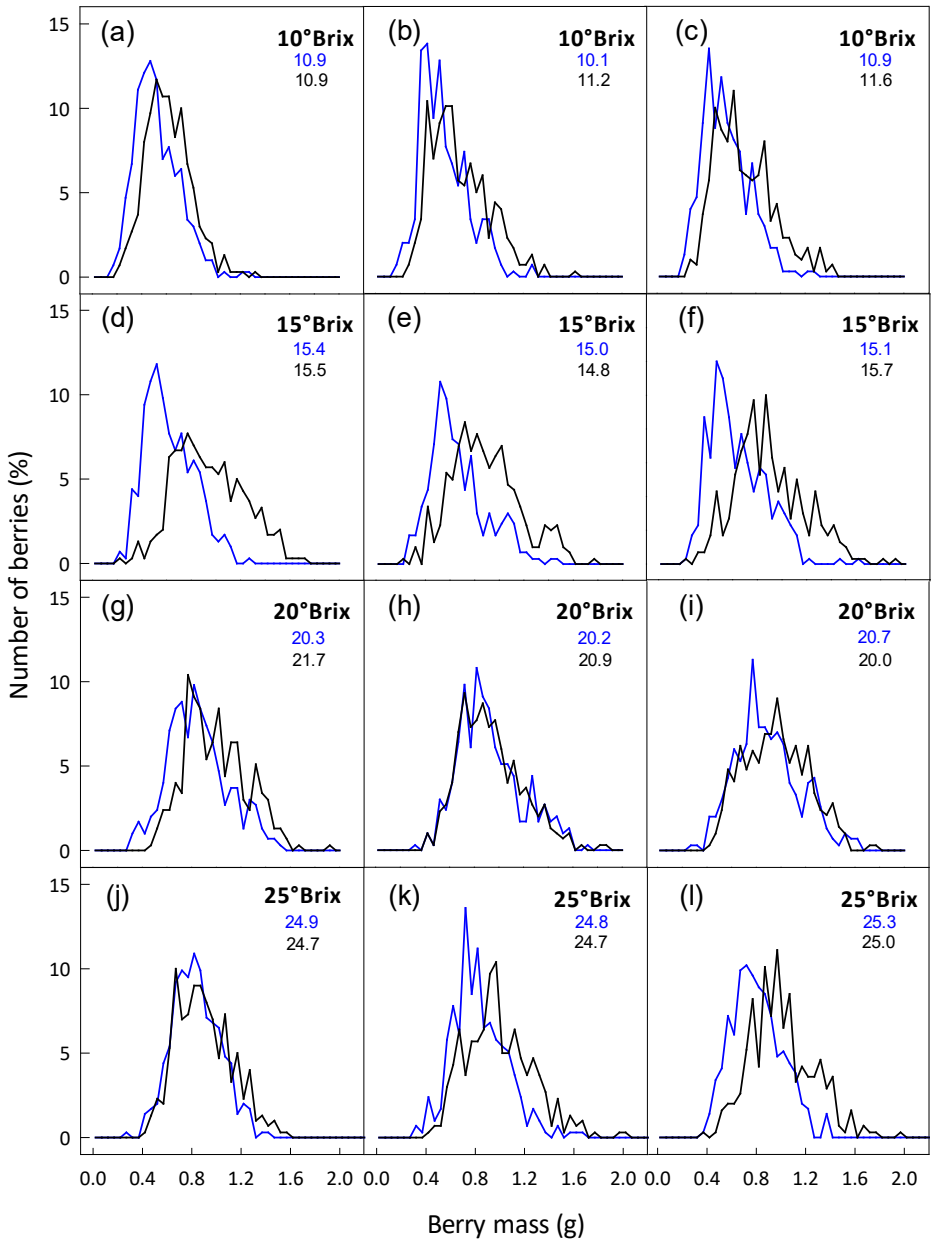


Figure 25. Berry fresh weight distribution profiles for control (——) and NAA-treated (——) berries at four stages of development, targeted to be at 10 °Brix, 15 °Brix, 20 °Brix and 25 °Brix. The precise mean TSS values are given underneath the nominal values (blue for Control, black for NAA-treated berries). T1, T2 and T3 denote the three treatments that occurred at 25, 16 and 0 days before veraison respectively. Berry weight was measured for an average of 296 individual berries for each curve and the weight values separated into classes with a range of 0.05 g. The number of berries for each class for each sample was plotted against the weight class.

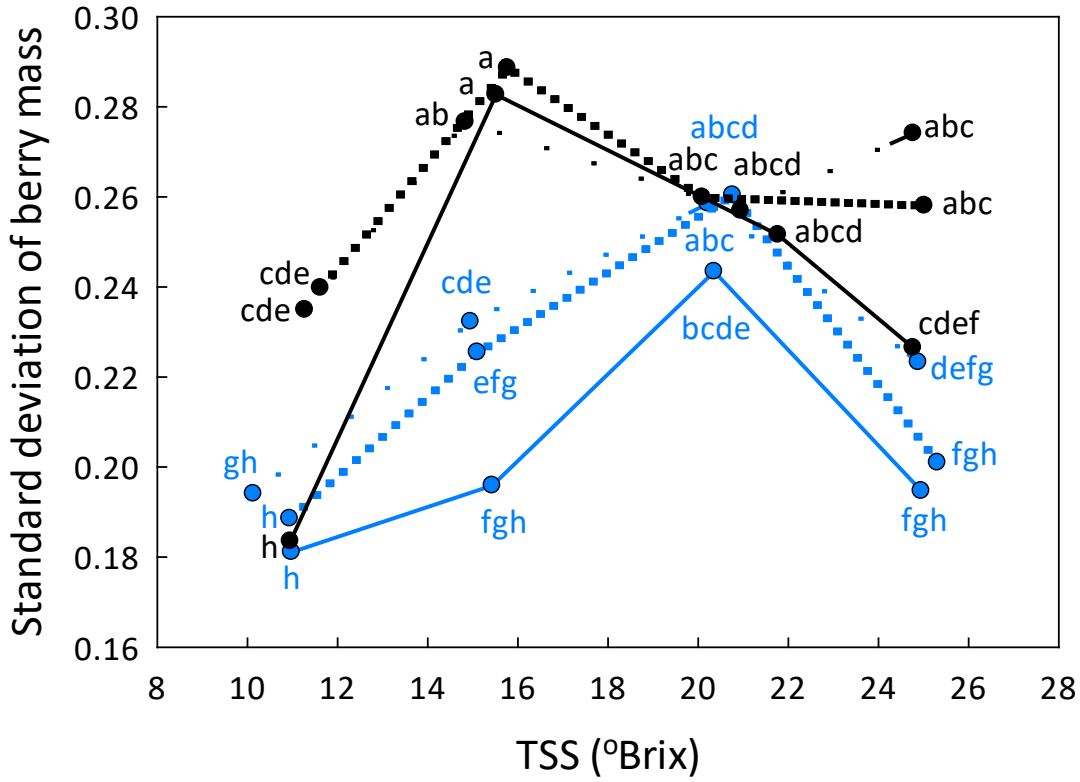


Figure 26. Plot of standard deviations of berry weight of Control treatment 1 (solid blue line), Control treatment 2 (dashed blue line), Control treatment 3 (dotted blue line) and NAA treatment 1 (solid black line), NAA treatment 2 (dashed black line) and NAA treatment 3 (dotted black line) berry populations during ripening. Values were calculated from an average of 296 berries for each curve and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test.

The distribution profiles for berry TSS (Figure 27) were in contrast to those seen for berry weight (Figure 25). At 15 °Brix, particularly for N1 and N2 treatments, the distribution of berry TSS was more synchronised than the distribution of Control fruit, as can be seen from the height of the respective peaks and the narrower distribution along the x-axis (Figure 27). This is confirmed by the smaller TSS standard deviation values for N1 and N2 berry populations at 15 °Brix (Figure 28). Overall, the TSS data confirmed the trend seen for the weight of berry populations as both Control and NAA-treated berries became more synchronous with regard to TSS as they approached 20 °Brix (Figures 27, 28).

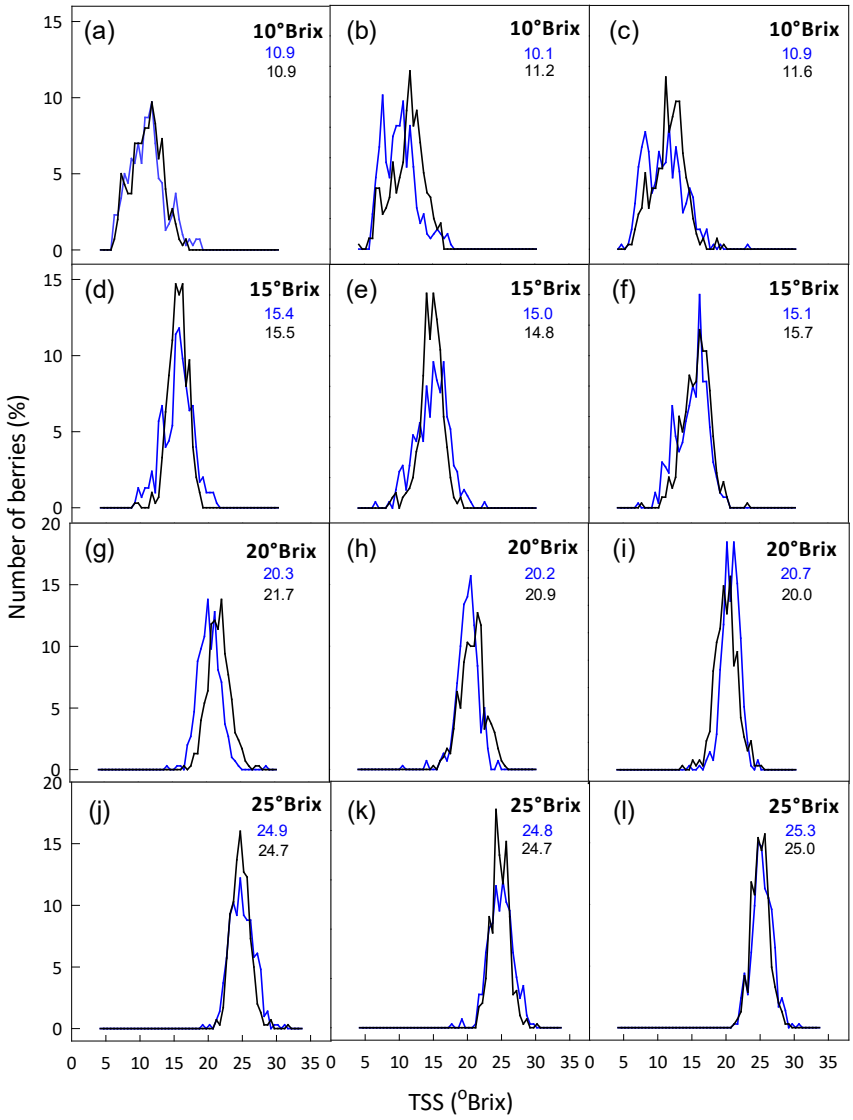


Figure 27. TSS (°Brix) distribution profiles for Control (——) and NAA-treated (——) berries at four stages of development, targeted to be at 10 °Brix, 15 °Brix, 20 °Brix and 25 °Brix, the precise, mean TSS values are given underneath the nominal values (blue for Control, black for NAA-treated berries). T1, T2 and T3 denote the three treatments that occurred at 25, 16 and 0 days before veraison respectively. TSS measurements were taken for an average of 296 individual berries for each curve and the values separated into classes with a range of 0.5 °Brix. The number of berries for each class for each sample was plotted against the TSS class.

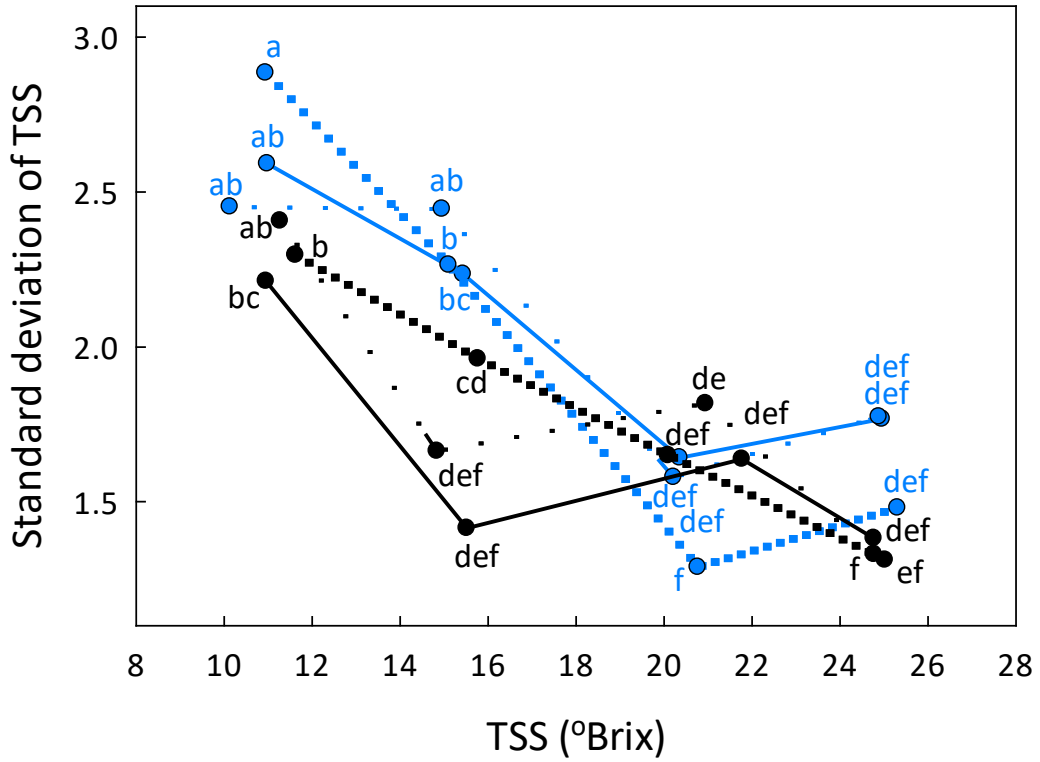


Figure 28. Plot of standard deviations of berry TSS (°Brix) levels of Control treatment 1 (solid blue line), Control treatment 2 (dashed blue line), Control treatment 3 (dotted blue line) and NAA treatment 1 (solid black line), NAA treatment 2 (dashed black line) and NAA treatment 3 (dotted black line) berry populations during ripening. Values were calculated from an average of 296 berries per curve and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test.

The phenomenon of NAA treatment altering the synchronicity of the ripening of the grape berry population was observed previously (Böttcher *et al.,* 2011b, 2012) and may mean that, in turn, the metabolism of these berries is better synchronised. Armstrong *et al.* (2021) sorted a berry population into different classes on the basis of density and showed that the level of heterogeneity had demonstrable effects on wine composition and sensory characteristics. While some variation in berry developmental stage might be useful in providing complexity to wine, too much heterogeneity may reduce consumer acceptance due to the presence of both green and over-ripe notes.

6.3.3.4 Changes to berry composition

A range of parameters was measured to further assess the influence of altering the onset of ripening on berry metabolism. Various macro- and micronutrients were measured in NAA-treated and Control berries at harvest. No significant difference between the NAA treatments and their respective Controls was observed for boron, calcium, copper, iron, magnesium, phosphorus, potassium, sodium, sulfur, and zinc (data not shown), while aluminium accumulation was lower in N2 (Figure 29). The limited difference in element accumulation between NAA-treated and Control fruit suggests that once ripening commenced,

elemental accumulation occurred much as it did in Control fruit. This suggests that vascular transport is largely unaffected by NAA-induced ripening delay.

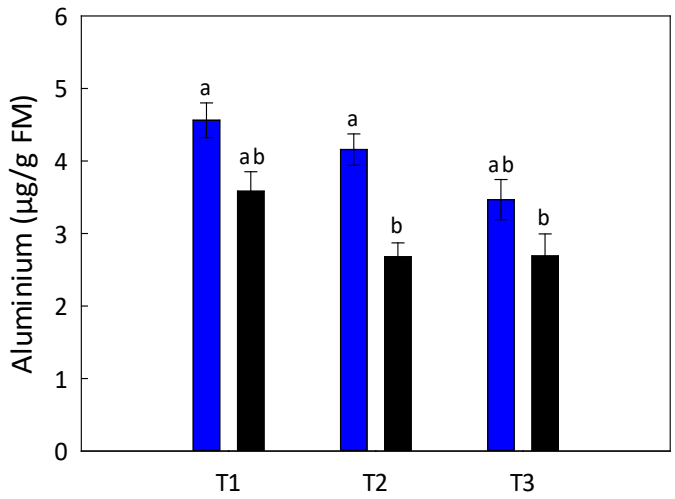


Figure 29. Aluminium concentration (µg/g fresh weight) for the three pairs of Control and NAA-treated fruit at harvest. T1, T2, T3 denote the three different timings. Lower case letters indicate if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Tukey's post hoc test.

Additional berry parameters were measured for Control and NAA-treated fruit at harvest (Table 14). One aim of the experimental protocol was to harvest the fruit for winemaking at as close to 25 °Brix as possible, to ensure the resulting wines were comparable. This was successfully accomplished as there were no significant differences between the berries from the six treatments for TSS, pH, glucose + fructose, or total YAN, indicating that NAA treatment did not significantly affect berry development apart from delaying ripening. This was further confirmed by the determination of anthocyanin concentration in NAA-treated and Control fruit at harvest, as no significant difference in this commercially important parameter was found between any of the treatments (Table 14).

Table 14. Compositional data for Control and NAA-treated berries at harvest. No significant differences detected by ANOVA analysis, ($P < 0.05$). PAN (primary assimilable nitrogen), YAN (yeast assimilable nitrogen).

Sample Date	Treatment	°Brix	pH	Gluc/Fruc (g/L)	PAN (mg/L)	Total YAN (mg/L)	Anthocyanin (A520 nm)
8 Mar	Control 1	24.9	3.98	199.24	136.62	202.15	49.0
	Control 2	25.1	3.97	197.69	156.16	223.39	43.5
	Control 3	25.3	3.96	201.67	158.24	222.45	47.2
21 Mar	NAA 1	24.7	4.06	204.77	169.14	214.78	46.3
29 Mar	NAA 2	24.9	4.06	209.41	167.28	200.64	42.2
4 April	NAA 3	24.8	4.10	213.61	147.04	200.90	41.8

6.3.3.5 General wine composition and wine volatile analysis

The finished Control and NAA wines exhibited small differences in ethanol concentration and TA but no significant differences for malic acid, glucose and fructose, volatile acidity and pH (Table 15).

Table 15. Ethanol, malic acid, total acid, glucose, fructose and volatile acidity content and pH of wines from Control and NAA-treated berries, as measured by OenoFoss analysis.

Treatment	Ethanol (% v/v)	pH	Total acid (g/L)	Malic acid (g/L)	Glucose/Fructose (g/L)	VA (g/L)
Control 1	14.67ab	3.61	5.13a	0.57	0.47	0.14
NAA 1	14.40abc	3.65	5.03ab	0.73	0.60	0.14
Control 2	14.80a	3.62	5.07a	0.50	0.53	0.13
NAA 2	14.33bc	3.68	4.97ab	0.60	0.50	0.10
Control 3	14.80a	3.62	5.20a	0.57	0.47	0.15
NAA 3	14.03c	3.67	4.83b	0.57	0.60	0.12

Values are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test.

Headspace analysis by GC/MS revealed that a total of 42 wine volatile compounds differed significantly in concentration for one or more Control/NAA treatment pairs, i.e. C1/N1, C2/N2, C3/N3 (Table 16, Table 17). Most of the fold-changes were small, only five of the differences in concentration were greater than two-fold. The largest fold change was for decanoic acid, which was 4.5-fold higher in N1 wine compared to C1 wine.

Table 16. Volatile compounds different (t-test, $P < 0.05$) in wines from Control or NAA-treated fruit.

Name	FC-T1†	FC-T2	FC-T3	Class	Odour
4-Methylpentan-1-ol			1.195	Alcohol	Nutty
3-Methylpentan-1-ol			1.297	Alcohol	Fusel
Hexan-1-ol	1.227	1.205	1.298	Alcohol	Green
Nonan-2-ol			-1.170	Alcohol	Green
Octanal		1.141		Aldehyde	Aldehydic, waxy
3,4-Dimethylbenzaldehyde		-1.239		Aldehyde	Naphthyl
Pentane-2,3-dione			1.165	Diketone	Buttery
Ethyl furan-2-carboxylate	-1.415			Ester	Balsamic
Diethyl butanedioate	-1.431			Ester	Fruity
Benzyl acetate			1.239	Ester	Sweet
Ethyl 2-hydroxy-3-phenylpropanoate	-1.822			Ester	Spicy
Ethyl but-2-enoate		-1.253		FA ester	Pungent, chemical
Ethyl (E)-hex-3-enoate		-1.370		FA ester	Sweet, fruity
Ethyl heptanoate		-1.688		FA ester	Fruity
Ethyl (E)-hex-2-enoate		-1.764		FA ester	Fruity
Propyl octanoate		-1.409		FA ester	Coconut
Propan-2-yl octanoate		-1.513		FA ester	Fruity
Methyl decanoate		-3.346		FA ester	Fatty
3-Methylbutyl octanoate		-1.419		FA ester	Sweet
Propyl decanoate		-1.484		FA ester	Waxy
Ethyl undecanoate		-1.536	-1.591	FA ester	Sweet
Ethyl 4-hydroxybutanoate	-1.492			FA ester	Caramel
Methyl dodecanoate	-2.962	-3.772		FA ester	Waxy
Ethyl dodecanoate		-1.366		FA ester	Sweet, waxy
3-Methylbutyl pentadecanoate		-1.499		FA ester	Waxy
Ethyl 3-hydroxyoctanoate	-1.396			FA ester	Winey
Ethyl tetradecanoate	-1.598	-1.383		FA Ester	Waxy
3-Methylbutyl dodecanoate	-1.667	-1.524		FA ester	Waxy
Ethyl 3-hydroxytridecanoate	-1.852		-1.316	FA ester	Waxy
Ethyl hexadecanoate	-1.861	-1.434		FA ester	Waxy
Decanoic acid	-4.510			FA	Rancid, fatty
3,5,5-Trimethylhexanoic acid		2.842		FA	Fruity
2-Pentylfuran			1.783	Furan	Fruity, green
γ-Butyrolactone			1.274	Lactone	Creamy
Eucalyptol		1.873		Monoterpene	Eucalyptus
p-Cymene			1.118	Monoterpene	Harsh, terpenic
β-Cyclocitral			1.259	Monoterpene	Tropical, fruity
1,1,6-Trimethyl-2H-naphthalene			-1.366	Naphthalene	Licorice
β-Damascenone		-1.469		Norisoprenoid	Sweet
β-Ionone		1.378		Norisoprenoid	Woody
β-Farnesene	1.209			Sesquiterpene alkene	Woody
2-Methylthiolan-3-one			-1.101	Thiolane	sulfur, fruity

† FC = fold change difference between the respective Control and NAA-treated wines, positive values = higher in Control wines, negative values = higher in wines from NAA-treated fruit.

Table 17. Kovats retention index and data source for assigning ID for compounds given in Table 16.

Name	Kovats†	ID‡
4-Methylpentan-1-ol	1298	A
3-Methylpentan-1-ol	1308	A
Hexan-1-ol	1335	A
Nonan-2-ol	1494	A
Octanal	1275	A
3,4-Dimethylbenzaldehyde	1780	B
Pentane-2,3-dione	1059	A
Ethyl furan-2-carboxylate	1593	A
Diethyl butanedioate	1648	A
Benzyl acetate	1699	A
Ethyl 2-hydroxy-3-phenylpropanoate	2000+	C
Ethyl but-2-enoate	1170	A
Ethyl (E)-hex-3-enoate	1283	A
Ethyl heptanoate	1313	A
Ethyl (E)-hex-2-enoate	1285	A
Propyl octanoate	1499	A
Propan-2-yl octanoate	1535	A
Methyl decanoate	1572	A
3-Methylbutyl octanoate	1641	A
Propyl decanoate	1702	B
Ethyl undecanoate	1719	A
Ethyl 4-hydroxybutanoate	1771	B
Methyl dodecanoate	1777	A
Ethyl dodecanoate	1821	A
3-Methylbutyl pentadecanoate	1840	A
Ethyl 3-hydroxyoctanoate	1858	B
Ethyl tetradecanoate	2000+	C
3-Methylbutyl dodecanoate	2000+	C
Ethyl 3-hydroxytridecanoate	2000+	C
Ethyl hexadecanoate	2000+	C
Decanoic acid	2000+	C
3,5,5-Trimethylhexanoic acid	1952	A
2-Pentylfuran	1223	A
γ-Butyrolactone	1601	A
Eucalyptol	1207	A
p-Cymene	1254	A
β-Cyclocitral	1589	B
1,1,6-Trimethyl-2H-naphthalene	1715	B
β-Damascenone	1789	A
β-Ionone	1904	A
β-Farnesene	1645	A
2-Methylthiolan-3-one	1499	A

†Calculated from retention relative to the retention of a series of n-alkanes (C8-C26). ‡A, Identity confirmed by matching weight spectra and LRI with that of authentic standards; B, Tentative assignment based upon comparison with NIST05 and Wiley Registry (7th edition) weight spectral libraries, and published retention times; C, Tentative identification of compound class suggested by comparison of weight spectra with those of related compounds

Only one compound, 1-hexanol, was significantly different across all three Control/NAA treatment pairs being lower in each of the NAA-treated wines compared to their respective Controls. The N2 treatment (24 compounds at significantly different concentration, five higher in C2, 19 in N2) had more effect on the concentration of volatiles than the N1 (13 compounds, 11 higher in C1, 2 in N1) and N3 (14 compounds, nine higher in C3 and five in N3) treatments. This indicates that the timing of application can influence berry metabolism and wine composition and that the second NAA application was at a stage of berry development that was most influential.

The largest group of compounds, 24 (56%) of the 43 that differed between wine from Control and NAA-treated berries, was the esters. Most of these, 20 examples, were fatty acid esters and all were higher in one or more wines made from NAA-treated berries. Ten esters (seven of which were fatty acid esters) were higher in N1 than C1, 17 fatty acid esters were higher in N2 than C2, one non-fatty acid ester was higher in C2 than N2, two fatty acid esters were higher in N3 than C3 and one non-fatty acid ester was higher in C3 than N3. Four of the 14 compounds that differed between C3 and N3 were alcohols but apart from these examples there were no other outstanding patterns for different classes of volatiles. Approximately one third of the fatty acid esters (Table 16) have odours described as 'waxy' and another third are described as 'sweet' or 'fruity'. The increase in fatty acid esters described as 'waxy' and 'fatty' could be a negative outcome for wines from NAA-treated fruit. However, the fold-changes for these esters were small and so these differences may not have substantially altered wine character. The other fatty acid esters were described by a range of descriptors including 'sweet' and 'fruity' (Table 16). Small increases in the ester fraction may add complexity to the wine and can alter the perception of fruity characters in the wine (Pineau *et al.,* 2009).

The modest changes in wine volatiles due to NAA treatment could arise from a range of causes. Auxin treatment has been shown to cause quite diverse changes in the berry transcriptome (Ziliotto *et al.,* 2012; Dal Santo *et al.,* 2020; He *et al.,* 2020, 2021,) and these changes might be responsible for altering berry metabolism. The production of wine volatile compounds or their precursors could be affected or changes to the matrix may have more subtle effects on yeast metabolism. It is possible that synchronisation of the TSS earlier in berry ripening of NAA-treated fruit may have subtly altered berry composition and may have played a part in the distinct volatile compound profiles seen for N1, 2, 3. In addition, due to the desire to harvest the fruit at the same TSS for winemaking, the NAA-treated fruit was harvested later than the Controls and the three NAA treatments were also harvested at different times to each other. Small differences in weather (Figure 23), water status and disease could therefore have impacted on the delayed-ripening fruit and resulted in the small differences observed in wine volatile composition.

Changes in fatty acid esters have been observed in other wines made from NAA-treated berries (Böttcher *et al.,* 2011b; Böttcher *et al.,* 2012; Davies *et al.,* 2015; this report). The fatty acid ester composition of wines made from Control and NAA-treated fruit is quite variable and while the reasons for this are unknown, the fold changes are low and may reflect minor differences in grape composition or fermentation conditions.

There were some compounds whose response in the current work was similar to that demonstrated in other experiments. For example, 3-methylpentan-1-ol, 4-methylpentan-1-ol, hexan-1-ol, β-cyclocitral and β-ionone were lower in concentration in wines from NAA-treated fruit in the current experiment and in wines from NAA-treated Cabernet Sauvignon and Shiraz fruit from another trial (this report). The norisoprenoid β-damascenone was higher in NAA wines in the current study (Table 16), in Riesling NAA

wines (Böttcher *et al.,* 2012) and in the juice of NAA-treated Cabernet Sauvignon berries, along with higher free and total norisoprenoids (He *et al.,* 2020).

Some of the differences observed between wines from Control and NAA-treated berries could result from reduced weight loss in the NAA-treated fruit. Chou *et al.* (2018) observed that two fatty acid esters and β-damascenone were at higher concentration in wines produced using non-shrivelled berries. Fatty acid esters and β-damascenone were at higher concentration in wine from NAA-treated fruit (Table 16), and these berries did not suffer weight loss that occurred in the Controls (Figure 21).

6.3.4 Conclusions

Here we have further investigated the efficacy of auxin treatments to delay the onset of veraison and hence harvest date. The closer to veraison the NAA treatments are applied, the more effective they are in delaying veraison and therefore fruit maturity. This means that adjusting the timing of treatment in relation to veraison is an option for controlling the length of the delay in harvest. Increasing the effectiveness of NAA in delaying ripening by applying it at the time of most effect will also mean that less NAA has to be applied, reducing costs and residues.

This work has shown a delay in the increase in TSS in berries from all three, differently timed, NAA treatments and the rate of sugar accumulation in these berries late in ripening was significantly lower than the rate observed for Control fruit at the same developmental stage. This slower rate of sugar increase in NAA-treated fruit near harvest was likely due to the lack of berry shrinkage which resulted in significantly larger berries at harvest for the N2 and N3 treatments compared to their Controls. A further result of this was that the NAA-treated berries stored more carbon resulting in a substantial yield increase, which is a beneficial side effect of the ripening delaying treatments for growers and winemakers.

The synchronicity of ripening in the berry population was affected, most obviously at 15 ^{o}Brix where N1, N2 berries were less synchronous in terms of weight but more synchronous in terms of TSS than the Controls. Even though the Control fruit became better synchronised with regard to TSS later in development, the NAA-treated fruit still remained more synchronised for a longer period.

The delay in ripening of NAA-treated fruit, particularly N2 and N3, moved the later stages of ripening into a cooler part of the season compared to the Controls, which may positively affect berry and wine composition. Any differences however, were quite subtle in terms of berry and wine composition. There was little difference in the basic berry parameters between the Controls and NAA-treated fruit and relatively small differences in wine volatile composition as only three out of 50, fold changes were greater than three and most were less than two.

These results show that the use of NAA can be an effective tool to tailor grape berry ripening/harvest timing with little effect on wine composition and with the benefits of more synchronous ripening and increased yield.

6.4 Auxin treatment of grapevine (*Vitis vinifera L.*) berries delays ripening onset by inhibiting cell expansion

6.4.1 Introduction

The previous experiments have shown that the application of the synthetic auxin 1-naphthaleneacetic acid (NAA) during the pre-veraison period delays the onset of grape berry ripening and hence delays harvest (Böttcher *et al.,* 2011a; Böttcher *et al.,* 2011b; Coombe and Hale 1973; Davies *et al.,* 2015, this report). The progression of development is arrested by auxin treatment, and it appears that the berries are held in a pre-veraison state until released from inhibition. The molecular mechanisms that lead to this delay are largely unknown. It seems likely that auxin treatment results in changes in the expression of auxin-related genes and consequent changes in a range of cellular processes. Interactions also occur with other hormone pathways (Ziliotto *et al.,* 2012). The timing of auxin application is critical, as applications much earlier than veraison, or at veraison, are not very effective in delaying ripening (Coombe and Hale 1973, this report). The lack of delay by the earlier treatment may be due to the removal of both the applied and endogenous auxin in a homeostatic response (Böttcher *et al.,* 2011a).

A better understanding of what controls berry ripening is of scientific and commercial interest. The work described below attempted to gain a more detailed understanding of the mechanism by which NAA delays ripening in order to discover any as yet unobserved effects and to better understand how a delay in ripening/harvest might be best achieved. The identity and function of auxin-responsive genes can provide valuable information about the mechanism responsible for the auxin-induced changes in berry development that leads to a delay of ripening. High throughput transcriptomic analysis is well suited to studying the changes in gene expression that result from perturbation of a PGR pathway as this methodology can determine the level of changes in transcription at a global level. The recent study of the effect of 2,4-dichlorophenoxyacetic acid on tomato ripening provides an example of how such an approach can be useful in fruit studies (Li *et al.,* 2016). In this work we have used a genome-wide transcriptomic approach to study changes in gene expression in grape berries treated with NAA at a time when berries are susceptible to a delay in ripening by auxins. The observed changes in gene expression revealed a large amount of cross-talk between hormone pathways and other changes in gene expression that are consistent with an auxin-induced ripening delay. Additional insights into the mechanism of NAA and the changes it induces in cell walls were gained through immunolabelling of cell wall components in berry thin sections. Understanding these changes offers insight into the mechanism by which auxins delay berry ripening and has practical utility in understanding the effects the delay has on berry metabolism.

6.4.2 Materials and methods

6.4.2.1 *Ex planta* berry induction assays

To study the effect of NAA treatment on berry RNA transcript levels experiments were undertaken during two different years. From a vineyard in the Adelaide Hills, 40 bunches at the pre-veraison stage were harvested from ten vines (*Vitis vinifera* L. cv. Shiraz), for each experiment. Bunches were harvested between 9-10 am and rapidly transported to the lab for use. For Experiment 1 (used for the array analysis), fruit was harvested on 21 Dec, 35 days before veraison. For Experiment 2 (used for the *in situ* immunolabelling of cell walls), fruit was harvested on 8 Jan, 19 days before veraison.

Randomly picked berries from the sampled bunches were sterilised in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, VIC, Australia) containing half a Milton antibacterial tablet L^{-1} (Milton Australia, Laverton North,

Victoria) for 10 min and washed three times with sterile nanopure water. Any berries showing physical defects were discarded. All of the following procedures were carried out in a laminar flow cabinet under sterile conditions. A thin transverse slice of each berry was cut off from the brush area to facilitate compound absorption. Twenty berries were then placed on each petri dish containing 25 mL of Gamborg media, 0.025% (w/v) casein hydrosylate, 0.8% (w/v) agar at pH 5.7-5.8, alone ('Control') or also containing 0.5 µM NAA (Gibo BRL Life Technologies, Grand Island, NJ, 'NAA-treated'). Each plate constituted a replicate and three replicates per treatment per time point were used. Berries were placed on the plates with the cut surface facing the agar; the plates were sealed with Parafilm and kept in the dark at room temperature. Berries were harvested at the times indicated below, wiped to remove agar, deseeded and frozen in liquid nitrogen. For *ex planta* NAA induction Experiment 1, berries were harvested at 3, 6, 12, 24 and 48 h after placement on the media, for *ex planta* Experiment 2, berries were harvested after 3, 24 and 48 h.

6.4.2.2 Microarray analysis

RNA prepared as above was tested for quality and quantity using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and a Bioanalyzer Chip RNA 7500 series II (Agilent, USA). Five micrograms of total RNA per sample was hybridised to a NimbleGen microarray 090818 (Roche, USA) as described by Dal Santo *et al.* (2013).

Multiclass statistical analysis of microarray (SAM) was carried out using TMeV v4.8 (mev.tm4.org/) with a false discovery rate (FDR) of 0.01%. A within-subjects t-test ($p<0.01$) was used to retrieve differentially expressed genes (DEGs) between Control and NAA-treated samples at each time-point.

6.4.2.3 cDNA synthesis and qRT-PCR

To confirm the array results and demonstrate that the changes in expression were similar between the two experiments, a selection of the genes that showed changes in gene expression upon NAA treatment was assayed by qRT-PCR. First-strand cDNA for qRT-PCR was synthesised as per Böttcher *et al.* (2013a).

6.4.2.4 Immunolabelling of thin berry sections

Segments of grape berries were dissected, fixed in 0.25% (v/v) glutaraldehyde, 4% (w/v) paraformaldehyde, and 4% (w/v) sucrose in phosphate buffered saline, pH 7.2 and dehydrated through an ethanol series then embedded in LR White resin according to Aditya *et al.* (2015). Sections (1 µm thickness) were prepared on a Reichert-Jung Ultracut ultramicrotome using a diamond knife and dried onto SuperFrost polysine coated 1 mm microscope slides (ThermoFisher Scientific, Australia). The regions of the berry that were sectioned and labelled are indicated in Figure 30.

Immunohistochemical analysis of cell walls was performed according to Wood *et al.* (2018). Primary antibodies were sourced from PlantProbes (UK) and included CBM3a, a type A carbohydrate-binding protein from *Clostridium thermocellum* that binds crystalline cellulose, LM19, a rat monoclonal antibody that detects homogalacturonan (HG), and LM20, rat monoclonal antibody that detects methyl-esterified homogalacturonan (meHG). For LM19 and LM20, primary antibodies were applied to sections followed by AlexaFluor 555 goat anti-rat IgG (H+L) secondary antibody (ThermoFisher Scientific, Australia). For detection of cellulose, a monoclonal anti-Histidine antibody (H1029, Sigma-Aldrich) was applied after a primary incubation with His-tagged CtCBM3a (Blake *et al.,* 2006). Slides were then incubated in a 1:100 dilution of AlexaFluor 488 anti-mouse IgG (Life Technologies) in PBS for 1 h. Samples were examined using a Zeiss AxioImager M2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam MRm camera.

Appropriate negative controls were included to verify the absence of cross-reactivity and eliminate false positives. Representative sections were also stained with the general stain Toluidine Blue (30 s with 0.01% [w/v] stain).

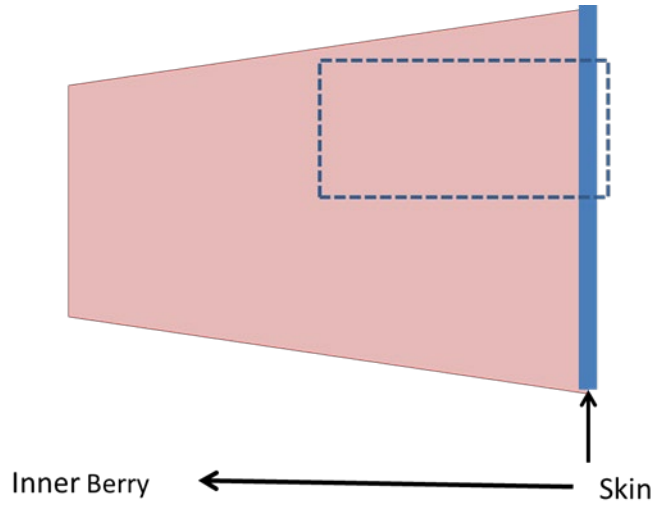


Figure 30. Schematic representing regions of berry thin sections imaged. The blue line indicates the location of the skin, the dotted rectangle indicates the location of sections used for immunolabelling.

6.4.3 Results and Discussion

6.4.3.1 The effects of NAA on grape berry gene expression change with time following treatment

A total of 1,892 genes (approximately 6.4% of the nearly 25,000 predicted grape genes represented on the array) were shown by Nimblegen array analysis to be differently expressed between Control and NAA-treated samples at one or more time-points (Figure 31). RNA-Seq analysis has determined that approximately 17,720 genes are expressed during grape berry development (Fasoli *et al.,* 2018), indicating that the transcription of nearly 11% of the genes expressed during berry development was significantly altered by NAA treatment in the current study. Considerably (approximately 2.8-times) more genes were up-regulated than down-regulated at 3 h; ratios for the other sampling times steadily decreased till reaching a ratio of 1.03 at the last time point (Figure 31). The early response to auxins is characterised by the rapid induction of members of the auxin/indole-3-acetic acid (Aux/IAA) transcriptional repressor, Gretchen Hagen3 (GH3) acyl acid amido synthetase and small auxin-up RNA (SAUR) gene families which are induced within a few minutes of auxin application (Hagen and Guilfoyle 1985; McClure and Guilfoyle 1987; Theologis *et al.,* 1985). The speed of movement of NAA through the berry tissue might be expected to be rapid, however, it is still probable that its distribution within the berry may be uneven, especially in the earlier stages of the time-course. In the work presented here, there was an up-regulation of members of the three, above mentioned, gene families at 3 h indicating that some early events had been captured.

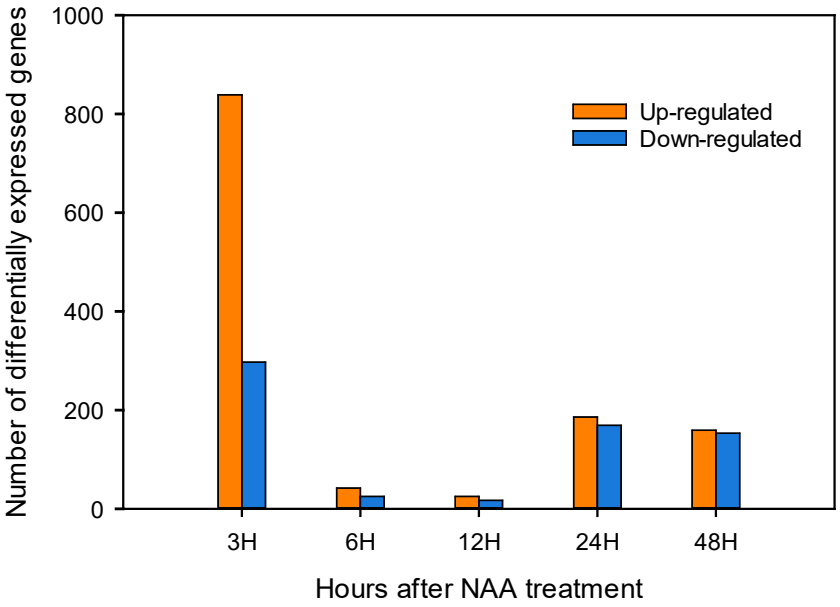


Figure 31. Global gene expression trends of NAA-treated *ex planta* grape berries. Number of genes significantly up-regulated or down-regulated at each time point.

Many genes were significantly differentially expressed at one single time-point, whereas only a few genes were differentially expressed at two or more time-points. The time-point at which most DEGs were found was 3 h (1,164 genes, Figure 31), considerably fewer genes were significantly altered in expression at the other time-points, particularly at 6 h and 12 h post-treatment.

To confirm that the validity of the array results, the expression of some genes was tested using qRT-PCR analysis (Table 18).

Table 18. Comparison of expression of a selection of genes up-regulated by NAA in two experiments using microarray and qRT-PCR analysis.

Gene ID	Expt. 1 Array[1]	Expt. 1 qRT-PCR[2]	Expt. 2 qRT-PCR[3]	Putative Function
VIT_11s0016g04810	12	3, 6, 48	3	BR catabolism
VIT_09s0002g05980	6, 12, 48	3, 6	no sig. diff	BR catabolism
VIT_07s0141g00270	6, 12, 24, 48	3, 6	3	auxin-induced
VIT-09s0002g05150	6, 12, 24	3, 6, 12, 48	3	IAA/Aux
VIT_07s0129g00660	6, 24, 48	3, 6, 24	3	auxin conjugation
VIT_04s0044g02010	6, 12, 48	3, 6, 24, 48	3, 24	GA synthesis
VIT_07s0005g03030	6, 12, 24	12, 48	3, 48	LBD protein

[1]Timepoints (h) at which a significant difference in expression between Control and NAA-treated fruit was detected by microarray analysis in Experiment 1.

[2]Timepoints (h) at which a significant difference in expression between Control and NAA-treated fruit was detected by qRT-PCR analysis in Experiment 1.

[3]Timepoints (h) at which a significant difference in expression between Control and NAA-treated fruit was detected by qRT-PCR analysis in Experiment 2.

6.4.3.2 Transcripts most highly modulated by NAA treatment

Genes whose expression had the largest fold change (positive or negative) when compared to the Control treatment might have a more significant effect on berry metabolism/development than genes exhibiting smaller changes. The list of the 30 most highly up-regulated genes (Table 19) contains a lateral organ boundary domain (LBD) gene which is a member of a large gene family of transcription factors in grapevine (Grimplet *et al.,* 2017). In other plants some LBD genes are regulated by auxin (Lee *et al.,* 2009; Paponov *et al.,* 2008; Sugawara *et al.,* 2015; Xu *et al.,* 2016) and have roles in the control of auxin transport and auxin signalling and in the inactivation of brassinolides (Turk *et al.,* 2003) which are involved in growth processes. One of the putative brassinolide inactivating genes, *BAS1*, is present in the top 30 list of genes whose expression was most highly induced by NAA suggestive of a decrease in the levels of castasterone, the active brassinosteroid in grape berries (Symons *et al.,* 2006). A gene encoding a 1-aminocyclopropane-1-carboxylate synthase (ACS), a key enzyme in ethylene biosynthesis, was also present in the top 30 most-induced list. The induction of ethylene biosynthesis by auxins has been previously observed in fruit (Coombe and Hale 1973; Trainotti *et al.,* 2007; Ziliotto *et al.,* 2012) and in grapes, the interaction between ethylene and auxin has been shown to be important in controlling ripening (Böttcher *et al.,* 2013c). Genes involved in auxin response and metabolism were not found in the 'top 30' list of induced genes but were present in the larger, complete list (data not shown). Five genes putatively associated with transport functions are represented in the top 30 up-regulated genes and transport-related genes are highly represented in the complete list (data not shown), suggesting that there is a change in transport functions.

Table 19. Top 30 up-regulated genes affected by *ex planta* NAA treatment. The darker the orange shading, the higher up-regulation.

		Fold change treated/untreated				
Gene ID	**Functional annotation**	**3h**	**6h**	**12h**	**24h**	**48h**
VIT_12s0035g01190	AMP-dependent synthetase and ligase	2.00	-1.01	1.03	-1.01	-1.02
VIT_07s0005g03030	Lateral organ boundaries Domain 16	-1.02	1.50	1.92	1.50	1.24
VIT_05s0020g00350	Chloroplast envelope membrane protein	1.83	1.31	1.08	-1.03	-1.07
VIT_12s0028g01910	Translation initiation factor 3 subunit K	1.76	1.09	1.12	1.03	-1.03
VIT_00s1667g00010	LEM3 (ligand-effect modulator 3) family protein	1.74	1.05	1.03	-1.01	-1.03
VIT_10s0003g05560	Pentatricopeptide (PPR) repeat-containing protein	1.62	1.01	1.05	-1.00	-1.01
VIT_04s0008g07230	No hit	1.61	1.05	-1.06	-1.20	1.12
VIT_19s0015g02330	AP-2 complex subunit mu-1	1.60	1.05	1.14	1.07	-1.02
VIT_17s0000g09060	Unknown protein	1.58	1.05	1.00	1.05	-1.04
VIT_10s0116g00060	NADH dehydrogenase subunit 7	1.57	1.20	1.04	-1.00	-1.08
VIT_01s0113g00530	No hit	1.56	1.02	1.01	1.07	-1.04
VIT_14s0006g00380	Nicastrin	1.56	1.05	1.05	-1.01	1.00
VIT_04s0023g02210	SAM dep carboxyl methyltransferase	-1.30	-1.13	-1.03	-1.91	1.55
VIT_11s0016g04810	BAS1 (PHYB activation tagged suppressor 1)	1.27	1.47	1.55	1.25	1.17
VIT_09s0018g01950	Cellulose synthase CSLC05	1.53	1.26	1.28	1.01	-1.03
VIT_17s0053g00160	Apyrase	1.51	1.16	1.10	1.06	1.01
VIT_18s0001g00910	Transportin-2	1.50	1.04	1.12	1.03	1.01
VIT_00s2117g00010	Bile acid:sodium symporter	1.50	1.06	1.11	-1.01	-1.00
VIT_06s0009g01520	Unknown protein	1.50	1.04	1.06	1.03	1.02
VIT_18s0075g00560	fatty acid multifunctional protein (MFP2)	1.49	1.01	1.03	1.01	-1.02
VIT_06s0004g02590	Carbonic anhydrase	-1.04	1.42	1.23	1.13	1.48
VIT_00s1437g00010	1-aminocyclopropane-1-carboxylate synthase	1.03	1.17	1.56	1.48	1.13
VIT_11s0016g02830	Gamma-glutamyltranspeptidase	1.48	1.03	1.00	1.04	1.03
VIT_00s0260g00070	Peptidyl-prolyl cis-trans isomerase, FKBP-type	1.48	-1.00	1.05	1.01	-1.01
VIT_01s0011g02520	EXS family protein/ERD1/XPR1/SYG1	1.02	-1.03	1.01	1.16	1.47
VIT_05s0020g03880	TSO1 (chinese for 'ugly')	1.47	1.00	1.13	-1.03	-1.02
VIT_07s0031g00040	Unknown protein	1.46	1.04	-1.02	1.05	-1.05
VIT_02s0025g04070	Phosphatidylinositol glycan, class U	1.46	1.05	1.05	1.01	-1.03
VIT_10s0071g01210	No hit	1.45	1.15	1.08	1.04	-1.01
VIT_08s0040g02300	ATP-dependent RNA helicase DDX52	1.44	1.06	1.06	-1.00	-1.01

The list of the 30 most highly down-regulated genes was dominated (nine out of the 25 with an identified function) by genes involved in various aspects of lipid metabolism. These include genes involved in fatty acid (FA) synthesis, wax and suberin synthesis and lipid breakdown and transport (Table 20). Similarly, in the complete list of NAA-modulated genes (data not shown) genes involved in lipid metabolism featured at a high frequency. Fatty acids are the starting material for a wide range of molecules involved in numerous processes including signalling in response to biotic and abiotic stresses, and polar auxin transport (De Bigault Du Granrut and Cacas 2016; Roudier *et al.,* 2010). The monomers that form cutin and suberin are also derived from FAs. The expression of numerous other FA synthesis and modification genes was altered by exposure to NAA but due to the complexity of the FA biosynthesis pathways it is difficult to propose a clear pattern for these changes.

Table 20. Top 30 down-regulated genes affected by *ex planta* NAA treatment. The darker the blue shading, the greater the down-regulation. The darker the blue colouration of boxes showing fold change, the larger the down-regulation. Asterisks (*) indicates genes putatively associated with lipid metabolism.

		Fold change treated/untreated				
Gene ID	**Functional annotation**	**3h**	**6h**	**12h**	**24h**	**48h**
VIT_11s0037g00320	Serine carboxypeptidase S10	1.02	1.07	1.05	-1.77	1.25
VIT_18s0001g14790	Lipase 3 (EXL3) family II extracellular *	1.02	1.07	1.20	-1.67	1.13
VIT_07s0031g01680	CYP86A1	1.03	-1.05	1.10	-1.65	1.49
VIT_05s0077g01650	Major allergen Pru ar 1	-1.09	1.05	1.16	-1.61	1.20
VIT_02s0025g01090	Unknown protein	-1.11	1.03	-1.16	-1.53	1.13
VIT_06s0080g00760	Unknown protein	1.03	-1.02	1.02	-1.51	1.44
VIT_06s0004g04000	Beta-ketoacyl-CoA synthase *	-1.07	-1.05	1.08	-1.46	1.15
VIT_01s0026g00570	Bet v I allergen	-1.03	1.01	1.03	-1.46	1.31
VIT_17s0000g02700	Alpha-dioxygenase 2 *	-1.10	1.01	1.09	-1.46	1.19
VIT_18s0001g02720	fatty acid elongation1 (FAE1) *	-1.24	-1.09	-1.08	-1.45	1.27
VIT_05s0077g00890	Pectinesterase family	-1.45	-1.03	-1.16	-1.14	1.06
VIT_13s0067g00400	Anthranilate synthase alpha 1	1.08	1.04	1.23	-1.43	1.33
VIT_05s0077g01670	Major cherry allergen Pru av 1.0202	-1.02	-1.01	1.02	-1.43	1.31
VIT_08s0058g01130	WNK5 (Arabidopsis WNK kinase 5)	-1.41	1.05	-1.10	-1.01	-1.07
VIT_18s0122g01470	CYP87A2	-1.39	-1.06	-1.08	-1.70	1.18
VIT_13s0047g00530	No hit	-1.39	-1.09	-1.11	1.11	1.02
VIT_04s0008g02610	FPF1 (flowering promoting factor 1)	-1.38	-1.01	-1.17	-1.10	-1.06
VIT_06s0061g00240	ABC transporter G member 7 *	1.03	-1.00	1.04	-1.38	1.20
VIT_12s0028g03480	Wax synthase *	-1.08	1.06	1.01	-1.37	1.16
VIT_14s0006g02990	3-ketoacyl-CoA synthase *	-1.11	1.00	1.07	-1.37	1.11
VIT_17s0000g00130	SEC14 cytosolic factor	1.01	1.01	1.09	-1.35	1.17
VIT_11s0118g00020	Exostosin-like	-1.35	-1.06	1.06	-1.10	-1.04
VIT_06s0009g03690	F-box domain containing protein	-1.34	-1.17	-1.11	-1.17	-1.13
VIT_00s0203g00170	Myb domain protein 92	-1.01	1.08	1.01	-1.34	-1.04
VIT_11s0037g01210	Eceriferum 1 (CER1 protein) Sterol desaturase *	1.06	-1.00	1.07	-1.34	1.20
VIT_02s0087g00210	Constitutive disease resistance 1	-1.33	-1.12	-1.12	-1.01	1.01
VIT_02s0087g00630	Alcohol oxidase	1.01	-1.01	1.08	-1.33	1.12
VIT_14s0066g00300	Unknown protein	-1.12	-1.01	-1.01	-1.33	1.21
VIT_04s0023g02160	Unknown protein	-1.06	-1.04	-1.32	-1.19	-1.09
VIT_08s0058g00990	Peroxidase	-1.05	-1.13	-1.06	-1.32	1.06

Three putative allergens are down-regulated as shown in the top 30 list (Table 20). These belong to the BET V 1-like superfamily of proteins (Radauer *et al.,* 2008), some members of which can bind and metabolise plant PGRs including brassinosteroids (Marković-Housley *et al.,* 2003), ABA (Klingler *et al.,* 2010) and cytokinins (Fernandes *et al.,* 2008; Pasternak *et al.,* 2006).

6.4.3.3 NAA-induced changes in the expression of genes involved in hormone metabolism and response

The application of NAA to berries induced changes in the expression of a wide range of genes involved in auxin response and metabolism, including the three classes of early response genes described above (Table 21 and data not shown). Six genes (*VviGH3.1-GH3.6*) encoding IAA amido synthetase enzymes are expressed in grape berries and the expression of four of them is increased by auxins (Böttcher *et al.,* 2011a; Böttcher *et al.,* 2010). These enzymes conjugate IAA to amino acids thereby inactivating it. In this work the expression of *VviGH3.1* and *VviGH3.2* was increased by NAA application (Table 21 and data not shown). The induction of *VviGH3.1* and *3.2* was maintained over several time-points in this experiment and this was probably due to the continuous exposure of the tissue to NAA. This is a common, homeotic response to decrease active auxin levels, not just in vegetative organs but also in fruit, including tomato (Li *et al.,* 2016), strawberry (Chen *et al.,* 2016) and orange (Ma *et al.,* 2014).

Table 21. NAA-induced changes in the expression of hormone pathway genes. Orange lettering = up-regulated expression, blue lettering = down-regulated expression

	Anabolism	Catabolism	Transport	Perception	Signalling
Auxin	IAA amino acid hydrolase 24h, IAA-amino acid hydrolase 6 3h	GH3.1 6,24h, GH3.2 6,24,48h, GH3.8 3h, IAA-glycosyltransferase 3h	PIN1 3h,24h, PIN3 3h, Auxin transporter-protein 4 24h, BIG 3h, WAG1 48h, Tornado 24h, RUS 3h		ARF6 3h, ARF9 3h IAA/Aux 6,12,24,48h IAA/Aux 12,48h IAA/Aux 12h IAA/Aux12 & 16 24h IAA/Aux17 12,24,48h IAA/Aux19 6,12,24h IAA/Aux29 12,24h IAA/Aux30 48h PLETHORA 1 3h, LPR1 24h, LBD1 24h, LBD11 24h, LBD16 24,24h
Ethylene	ACS 3h ACS 24h ACS 24h ACS 48h ACO1 3h ACO2 48h			EIN4 3h, 24h, ETR1 3h, CTR1 24h	ERF003 48h, ERF105 3h, ERF105 24h, ERF113 48h, EIN3-binding F-box protein 1 & 2 3h, APETALA2 6-like 3h, TSRF1 (2 genes) 48h
ABA	Violaxanthin de-epoxidase 3h				HAB1 3h, TUBBY like protein 3 48h, OST1 3h, PAPA1 3h, LRK10 6h, LRK10 48h
GA	GA20 oxidase 3h, GA20 oxidase 6,12,48h	GA2 oxidase 24h, GA response protein Spindly 3h			Della RG1 (repressor) 3h, DWARF and delayed flowering 1 24h
BR	24-sterol C-methyltransferase 3h	BAS1 12h, CAS26OH 6,12,48h		BRI 3h, BRI1-KD 3h	Protein kinase CDG1 3h, Protein kinase CDG1 24h
CK		Cis-zeatin O-beta-D-glucosyltransferase (2 genes) 48h			ARR9 (type A, VviRR39) 6h
JA	Jasmonate o-methyl transferase 3h, SAM dep. carboxyl methyl transferase 48h, 12-oxophytodienoate reductase 2 3h				TIFY9 48h
SA	SAM dep. salicylic acid carboxyl methyltransferase (2 genes) 24h, Isochorismate synthase 1 3h			NPR1 3h	

A number of Aux/IAA genes (involved in controlling the expression of auxin-induced genes) were induced by NAA, the transcript levels of eight out of the nine were significantly higher at 24 and/or 48 h after the auxin treatment commenced (Table 21 and data not shown). *VvIAA19* was expressed early in berry

development and post-veraison (both are periods of berry expansion) and is thought to be a positive regulator of plant growth (Kohno *et al.,* 2012). The change in expression of some of the above mentioned Aux/IAA genes may be part of a homeostatic response to elevated auxin levels, similar to the observed induction of GH3 genes. The induction of expression of Aux/IAA genes as a result of auxin application has also been observed in other fruit including tomato (Li *et al.,* 2016), papaya (Liu *et al.,* 2017), strawberry (Chen *et al.,* 2016) and orange (Ma *et al.,* 2014).

Auxin response factors (ARFs) can either promote or supress auxin-related gene expression (Li *et al.,* 2016). Two ARFs were altered in expression following NAA application to grape berries (Table 21). The expression of some other auxin-related transcription factors was also altered by exposure to NAA e.g. a putative PLETHORA 1 homolog and *LRP1* (lateral root primordium 1) were down-regulated.

The expression of five putative auxin-responsive SAUR genes was altered by NAA treatment. SAURs are involved in the regulation of a wide range of processes including auxin transport, cell expansion, leaf growth and senescence, root growth and development and tropic growth (Ren and Gray 2015). The expression of two grape SAUR genes was down-regulated by NAA and that of three others was up-regulated. A similar, mixed response of SAUR gene expression was observed in tomato fruit at the mature green stage in response to 2,4-dichlorophenoxyacetic acid (Li *et al.,* 2016).

6.4.3.4 Auxin transport

The expression of a number of genes putatively involved in auxin transport was altered by exposure to NAA. Two PIN homologs (auxin efflux transporters) were up-regulated at 3 h post NAA treatment (data not shown). One of these, VIT_01s0011g04860, was classified as a member of the family of 'long PIN' transporters which are located in the plasma membrane and are involved in polar auxin transport (Křeček *et al.,* 2009). In the current work VIT_05s0062g01120 expression was increased at 3 h but decreased at 24 h post NAA treatment. These changes in gene expression indicate that as part of a homeostatic response to NAA, cells may react by increasing auxin efflux and reducing IAA influx. Note that NAA diffuses readily into the cell (Delbarre *et al.,* 1996) and does not require active transport.

The expression of other genes involved in auxin transport was also up-regulated by NAA e.g. a *WAG1* gene homolog, a kinase important for apical PIN localisation (Dhonukshe *et al.,* 2010), and activation (Zourelidou *et al.,* 2014) and a *RUS1* (Root UVb sensitive 1) homolog, involved in polar auxin transport in *Arabidopsis* (Yu *et al.,* 2013), was up-regulated at 3 h. Changes to the expression of genes involved in cytoskeletal function (data not shown) may also affect PIN localisation. Taken together, the above changes indicate a reprogramming of the auxin transport systems to increase auxin efflux in response to sustained exposure to 0.5 µM NAA.

6.4.3.5 Ethylene-related

After auxin-related genes, the largest group of hormone-related genes that responded to NAA were those involved in ethylene metabolism/signalling (Table 21 and data not shown). Transcripts of three putative ACC (1-aminocyclopropane-1-carboxylate) synthase genes (ACS) were significantly up-regulated, while one was slightly down-regulated at 3 h. ACS was similarly induced by IAA in *Arabidopsis* (Goda *et al.,* 2004; Sugawara *et al.,* 2015). The expression of two putative ACC oxidase genes (ACO) was induced by NAA. These changes in expression are suggestive of an increase in ethylene biosynthesis. It has been shown previously that the application of an auxin to grape berries produced an increase in ethylene evolution (Coombe and Hale 1973).

The expression of two EIN3-binding F-box genes which regulate the ethylene signalling cascade by negatively modulating the stability of EIN3 and EIL1 proteins (Gagne *et al.,* 2004) was down-regulated by NAA (Table 21 and data not shown). The down-regulation of the ethylene receptor *ETR1* is also consistent with an increase in ethylene signalling as a reduction in ethylene receptor molecules in cells should increase the sensitivity to ethylene. The expression of a *CTR1* homolog, which represses the ethylene signalling pathway, was down-regulated at 24h which also indicates an increase in sensitivity to ethylene. The expression of putative ethylene-responsive TF's (ERFs) and putative ethylene-inducible TSRF transcription factors was also altered by NAA (Table 21 and data not shown). Overall, the changes in gene expression described above indicate that an increase in ethylene synthesis and signalling occurs upon auxin application. The role of ethylene in grape berry is complex and there is evidence that an early application of ethylene can delay ripening, most probably through an induction of auxin biosynthesis (Böttcher *et al.,* 2013b; Hale *et al.,* 1970). The application of Ethrel or ethylene nearer to veraison can promote ripening (Böttcher and Davies 2012; Hale *et al.,* 1970; Szyjewicz *et al.,* 1984).

6.4.3.6 Abscisic acid-related

The response of abscisic acid (ABA)-related genes to auxin exposure is modest in comparison to the response of the auxin and ethylene pathways. It seems that the response of the ABA pathway to NAA is both mixed and limited. As ABA is a positive regulator of berry ripening (Wheeler *et al.,* 2009) it would be surprising if ABA signalling was greatly up-regulated by auxins.

6.4.3.7 Gibberellic acid-related

Two putative grape gibberellin 20 oxidase genes (GA20ox) were considerably up-regulated by NAA (Table 21 and data not shown). These enzymes are involved in the synthesis of active gibberellins (Sponsel and Hedden 2010). In contrast, a *GA2-beta-dioxygenase 7* homolog, which catalyses beta-hydroxylation of some active GAs, greatly reducing their activity, was down-regulated. Together these changes in gene expression suggest that NAA may result in increased GA concentrations. Auxins and GAs have a close association during processes involving cell division/cell expansion and both are at high concentrations during the early stages of berry development, decreasing to be low before the initiation of berry ripening (Böttcher and Davies 2012), so the observed changes in GA-associated gene expression may be part of the mechanism that maintains the pre-veraison status of berries.

6.4.3.8 Brassinosteroid-related

The expression of two putative castasterone 26-hydroxylase genes, encoding an enzyme that inactivates castasterone (Turk *et al.,* 2003), was up-regulated by NAA treatment (Table 21 and data not shown). It has been shown that castasterone concentration increased from veraison, that epi-brassinolide application pre-veraison advanced ripening and an inhibitor of brassinosteroid biosynthesis delayed it (Symons *et al.,* 2006). As the concentration of castasterone is already low at the developmental stage at which NAA was applied it may be that the up-regulation of the castasterone 26-hydroxylase genes will further delay the ripening associated increase in castasterone and may therefore be a factor in the auxin-induced ripening delay. The transcriptional changes to ABA and brassinosteroid pathways, hormones known to be positive regulators of ripening (Böttcher and Davies, 2012), align with the delaying of ripening by auxin application as there is not a significant up-regulation of the ABA pathway and two genes encoding enzymes that catabolise brassinosteroids are significantly up-regulated.

6.4.3.9 Other hormones

A few genes associated with cytokinins, jasmonates and salicylic acid were altered in expression by exposure to NAA (Table 21 and data not shown). The extent of these changes was quite modest but indicates that some up-regulation of the biosynthesis of these hormones may have occurred.

It is difficult to distinguish which of the changes in transcription were a direct effect of exposing pre-veraison grapes to NAA and which were secondary events due to cross-talk between hormone pathways, but this cross-talk is likely to be responsible for a significant proportion of the observed changes. Despite this cross-talk, the delaying effect appears to be auxin specific. As described previously, the delay caused by an application of ethylene early in fruit development may be due an increase in IAA accumulation (Böttcher *et al.,* 2013). The application of other hormones to pre-veraison grape berries does not have a dramatic effect in delaying ripening. Only when the auxin concentration decreases e.g. by catabolism by GH3 enzymes, is the restriction on progression into the ripening phase lifted and berry development recommences.

6.4.3.10 NAA treatment modulates the expression of cell wall-associated genes

In general, the expression of genes involved in cell wall synthesis was up-regulated by NAA treatment, while those likely to be involved in degradation, particularly those involved in the modification and breakdown of pectins, were down-regulated. Genes encoding proteins involved in the synthesis of cellulosic cell wall components that were up-regulated include putative cellulose synthase (*CesA*) and cellulose synthase-like (*Csl*) genes. These genes encode enzymes that are putatively involved in the synthesis of primary cell wall cellulose (CesA, CslD2/3), xyloglucan (ClsC5) or possibly mannan (CslD2/3) (Cocuron *et al.,* 2007; Verhertbruggen *et al.,* 2011, Schwerdt J pers. comm.). Fasoli *et al.* (2016) has previously reported that the expression of four genes annotated as cellulose synthases was down-regulated in Corvina skin during ripening, while two were slightly up-regulated. The up-regulation of *CesA* and *Csl* genes by NAA could therefore be viewed as maintaining cell wall synthesis in the pre-veraison state. This is further supported by the up-regulation by NAA (data not shown) of an endo-1,4-β-glucanase (Korrigan-like) thought to be required for cellulose synthesis (Maloney *et al.,* 2012). Two endo-1,4-β-glucanases were down-regulated in Corvina skin (Fasoli *et al.,* 2016) and in tomato and strawberry fruit during ripening (Chen *et al.,* 2016; Li *et al.,* 2016), which again supports the view that cell wall synthesis was continuing in NAA-treated fruit as it would in the pre-veraison state. Changes to the transcript levels of cytoskeletal genes may also affect cell wall synthesis.

Genes involved in xyloglucan metabolism were down-regulated including, two xyloglucan endotransglucosylase (XET) genes that are thought to promote cell expansion through breaking and reassembling the xyloglucan cross-links between cellulose microfibrils (Liu *et al.,* 2007), an exostosin (xyloglucan galactosyltransferase KATAMARI 1) and an α-1,6-xylosyltransferase (involved in xyloglucan biosynthesis, Vuttipongchaikij *et al.,* 2012). The expression of three XET genes was down-regulated by IAA in *Arabidopsis* (Goda *et al.,* 2004). XET gene expression was up-regulated during berry ripening in Shiraz and Muscat Gordo Blanco berries (Nunan *et al.,* 2001), Corvina skin (Fasoli *et al.,* 2016) and the berries of a *Vitis vinifera* × *V. labrusca* L. cross, Kyoho (Ishimaru and Kobayashi 2002).

Of particular interest are the genes whose changes in expression might suggest that NAA resulted in a reduction in pectin catabolism. The expression of five genes encoding putative pectinmethylesterase enzymes (PMEs) was down-regulated (data not shown). PMEs catalyse the de-esterification of methyl-esterified D-galactosiduronic acid units in pectin, which makes the pectin more susceptible to degradation

enzymes such as polygalacturonase (PG, Fischer and Bennett 1991). Another esterase, pectinacetylesterase was up-regulated. This enzyme deacylates pectin and makes it less vulnerable to digestion by pectinase (Gou *et al.,* 2012). The expression of genes encoding pectin-degrading enzymes was mainly down-regulated. Of the four PG genes (their product hydrolyses the alpha-1,4 glycosidic bonds between galacturonic acid residues) whose expression was altered, three were decreased in expression while one was up-regulated (data not shown). NAA treatment of strawberry also down-regulated PG mRNA, protein and enzyme activity levels (Villarreal *et al.,* 2009). The expression of one PG gene increased after veraison in Cabernet Sauvignon skins (Deytieux-Belleau *et al.,* 2008) and there was some expression in Gordo and Shiraz berries during ripening (Nunan *et al.,* 2001) but in contrast two PG genes were down-regulated in Corvina berry skin during ripening (Fasoli *et al.,* 2016). The role of PG in grape berry ripening is not clear as no enzyme activity was detected in berries by Nunan *et al.* (2001) or in skins by Deyteux-Belleau *et al.* (2008). However, grapes are high in phenolic compounds that can inhibit enzyme activity upon extraction and so a null result may not be conclusive.

The depolymerisation and solubilisation of pectin have been reported to occur during grape berry ripening (Nunan *et al.,* 1998; Yakushiji *et al.,* 2001). Taken together, the changes to the expression of pectin modifying gene expression resulting from NAA treatment could indicate that the cell wall changes associated with ripening might be delayed. In addition, the expression of a gene putatively involved in pectin biosynthesis, polygalacturonate 4-alpha-galacturonosyltransferase *GAUT11*, was up-regulated at 3 h.

6.4.3.11 Changes in cellulose and homogalacturonan epitopes in NAA-treated berries

To investigate whether compositional changes mirrored the observed changes in the transcript levels of cellulose and pectin-related genes, sections of grape berries were analysed by thin-section immunolabelling using the CMB3a carbohydrate-binding module (detects crystalline cellulose) and LM19 and LM20 antibodies (which detect homogalacturonan (HG) and methyl homogalacturonan (meHG) epitopes respectively). Berry segments were dissected from Control and NAA-treated berries and sectioned to reveal cellular details from the skin (exocarp) through to the mesocarp and endocarp (Figure 30).

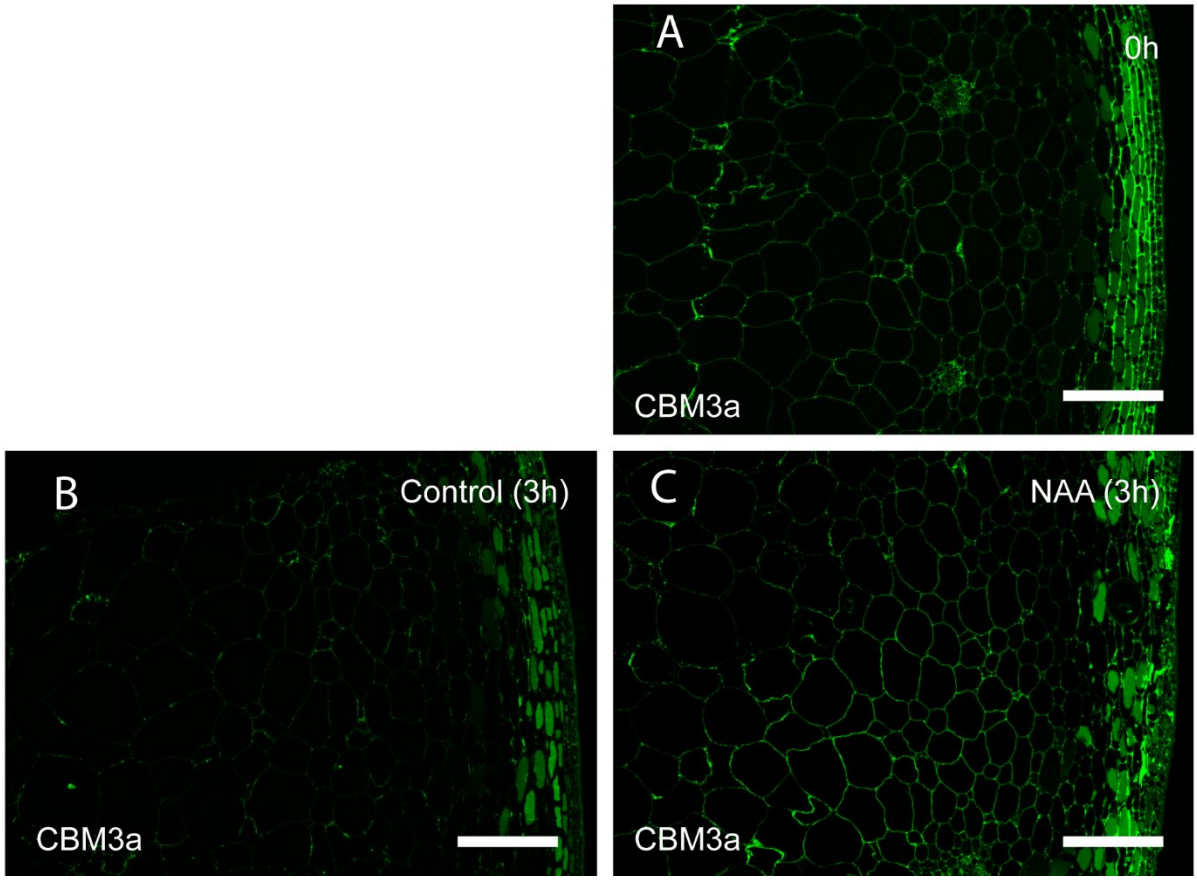


Figure 32. CBM3a (cellulose) labelling in Control and NAA-treated *ex planta* grape berries. CBM3a immunolabelling of cell wall components in thin sections of grape berries. All panels are arranged to show a similar orientation. Labelling is indicated by bright green fluorescence, A Berry prior to treatment at 0 h. B Control berry at 3 h. C NAA-treated berry at 3 h. Bar = 150 µm.

Labelling with the CBM3a protein decreased in the Control exocarp and mesocarp tissues from 0 h (Figure 32). At 3 h the signal in the exocarp of both treatments was much reduced. However, the labelling was higher in the NAA-treated fruit compared to the Control. At higher magnification it was observed that some labelling persisted in the pericarp of NAA-treated berries at 24 h and 48 h (data not shown). The response to NAA in the mesocarp was quite different for Control and NAA-treated berries. The labelling was maintained, or even increased, in NAA-treated berries at 3 h and was much more evenly distributed around each cell, after 3 h it declined to be similar to the labelling found in the Control samples. The higher level of cellulose resulting from NAA application could be due to reduced hydrolysis but the observed increase in cellulose synthase gene expression suggests that some additional synthesis occurred.

The LM20 antibody was used to detect meHG (Figure 33). In Control berries prior to treatment, labelling was weak or absent in the outermost layer of the exocarp (i.e. the epidermis) but was abundant in the walls of sub-epidermal exocarp cells (Figure 33A). In mesocarp cells, labelling was concentrated at cell junctions (Figure 33A). In Control berries, LM20 epitopes decreased in the exocarp over time (Figure 33B, and data not shown) and by 48 h were barely detected. Despite this, label was detected in mesocarp cells of Control berries throughout the time-course, although the intensity was weaker at 24 h and 48 h than

that at 0 h and 3 h. After 3 h of NAA-treatment (Figure 33B), LM20 labelling appeared indistinguishable from Control berries at 0 h and 3 h. However, clear differences were observed at 24 h and 48 h (data not shown). In contrast to Control berries, LM20 epitopes were abundant in the exocarp tissues of NAA-treated berries throughout the timecourse. Labelling efficiency at 48 h was similar to that in Control berries prior to treatment and clearly different to Control berries at 48 h. Contrasting this, signal was barely detected in mesocarp cells of NAA-treated berries at 24 h and 48 h, while Control berries reproducibly showed some labelling (data not shown).

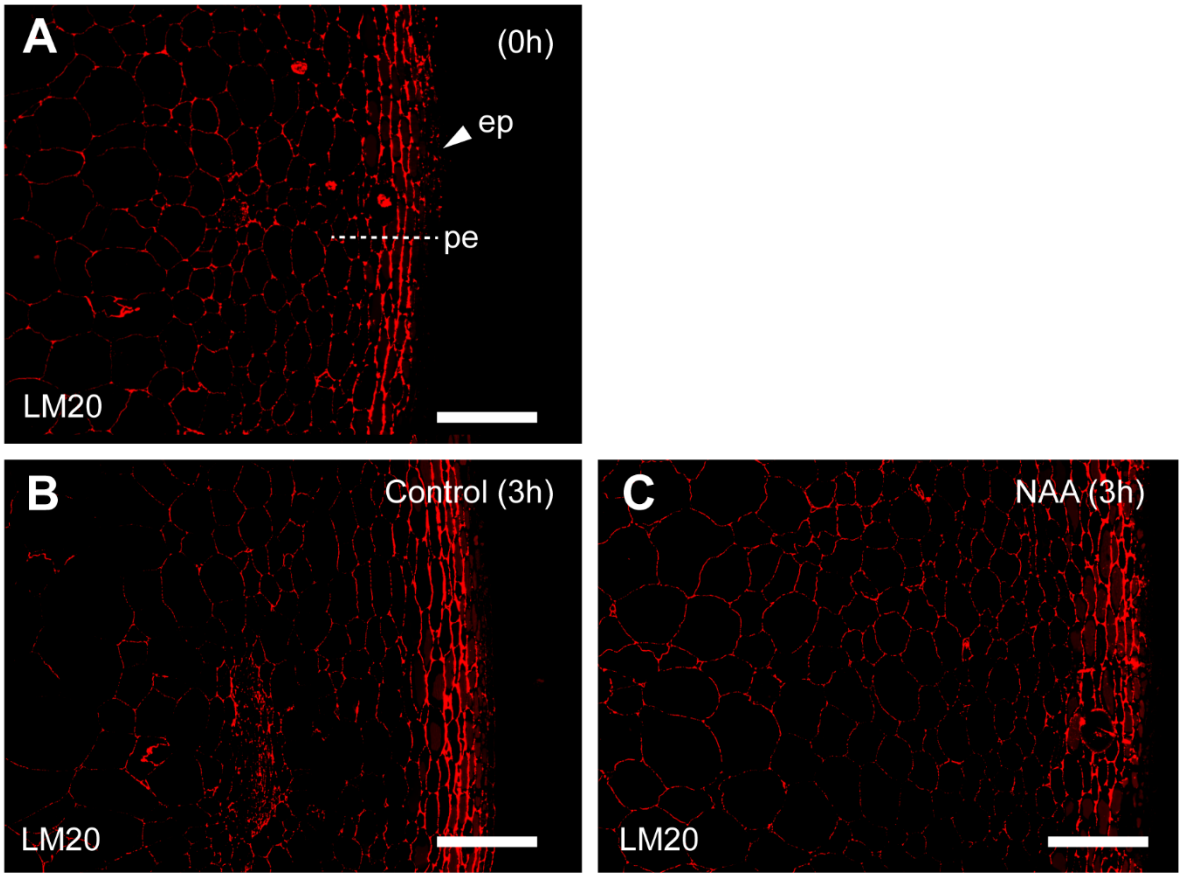


Figure 33. LM20 (meHG) immunolabelling in Control and NAA-treated *ex planta* grape berries. LM20 immunolabelling of methyl-esterified homogalacturonan in thin sections of grape berries. A Section of a Control berry (0h) showing the position of the epidermis (arrow) and pericarp. Labelling is indicated by bright red fluorescence predominantly in the pericarp, but with some labelling in mesocarp cells. B Control berry at 3 h. C NAA-treated berry at 3 h. ep, epidermis, pe, pericarp. Bar = 150 µm.

In summary, the LM20 (meHG) epitope showed differential accumulation in distinct tissues of *ex planta* berries over time. Cell walls of the exocarp show a decrease in LM20-immunolabelling over time, while cell walls in the mesocarp retain some meHG epitopes. Treatment of berries with NAA had a pronounced effect, apparently blocking the decrease of meHG in the exocarp, while enhancing the removal of the epitope in mesocarp cells after 3 h. The maintenance of exocarp LM20 labelling could potentially affect the binding sites of receptors, involved in controlling cell wall composition, in a tissue-specific manner. The *Arabidopsis* receptor protein FERONIA (FER; Feng *et al.,* 2018), which acts as a sensor of cell wall

integrity, binds directly to pectin. Another member of the *FER* (*CrRLK1*) family named *ERULUS* (ERU) is regulated by auxin at transcriptional and post-translational levels and is a negative regulator of PME activity in root hairs (Schoenaers *et al.,* 2018). These findings suggest that a FER-ERU feedback mechanism involving pectin methyl-esterification and pectin-dependent receptor binding may alter cell wall metabolism and cell expansion (Barbez *et al.,* 2017; Schoenaers *et al.,* 2018).

Based on labelling intensity, LM19 (HG) epitopes appeared to be more prevalent in berries compared to LM20 (meHG). In pre-veraison berries, LM19 labelling was detected in most cell walls, particularly in the exocarp and endocarp (Figure 34A). In Control berries, labelling appeared to gradually decrease in most cell walls over time. At 48 h, labelling was absent in the exocarp but some residual label was detected in the endocarp and adjoining mesocarp cells (data not shown). Clear differences were observed in sections of berries treated with NAA. At 3 h after application (Figure 34C), the pattern and intensity of LM19 labelling appeared unchanged relative to the 0 h Control (Figure 34A). Labelling was uniformly distributed in cell walls across the berry, which contrasted with Control berries at 3 h that already showed diminished labelling in some mesocarp cells. LM19 epitopes were detected at 24 h (Figure 34E) after NAA treatment and were particularly abundant in the exocarp. Some patches of mesocarp cells showed reduced labelling, but this was less obvious than in Control samples. In summary, HG levels in *ex planta* berries appear to decrease in most cell types over time. However, NAA-treatment delayed or inhibited the removal of these epitopes, particularly in exocarp cells.

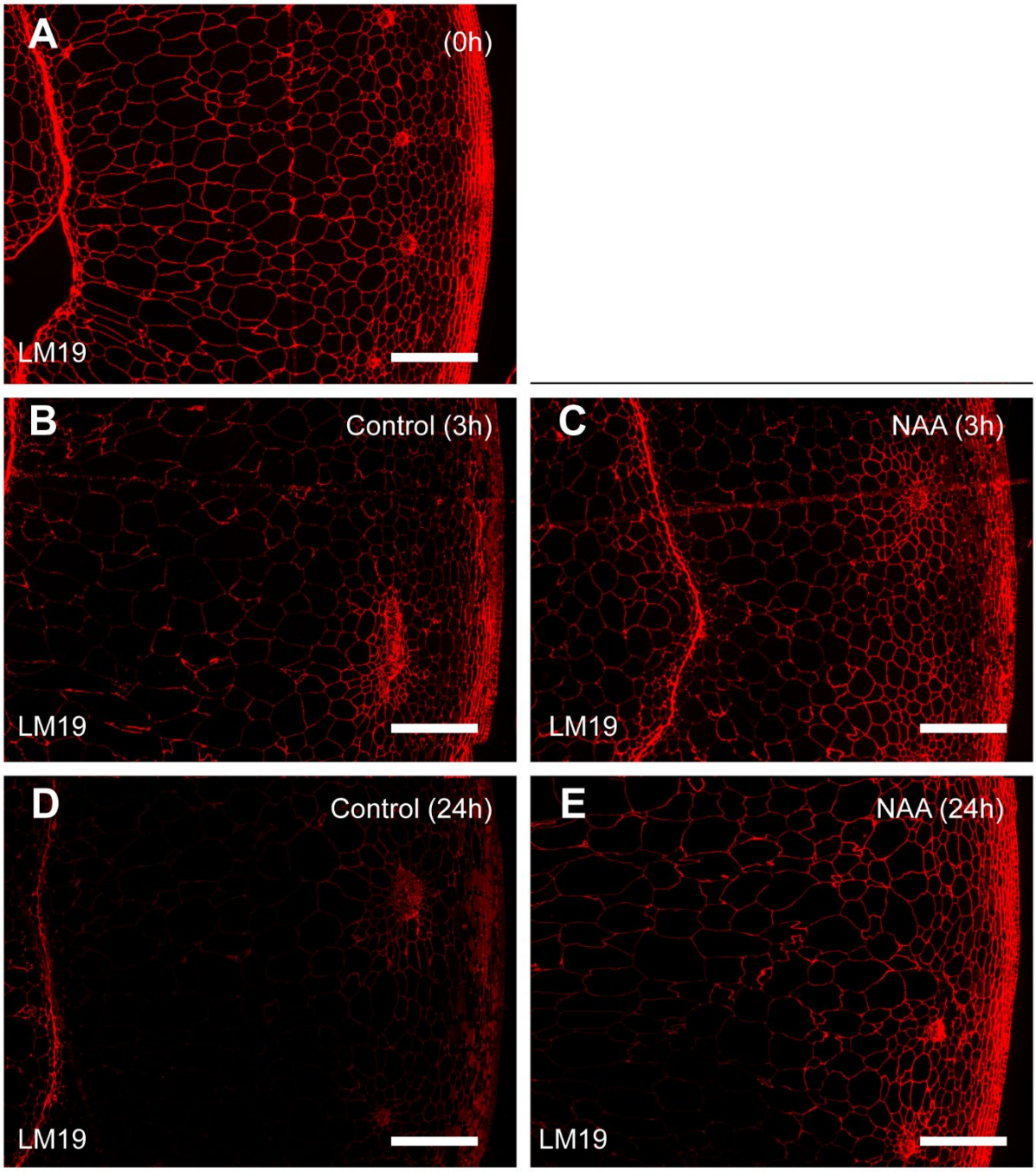


Figure 34. LM19 (HG) immunolabelling in Control and NAA-treated *ex planta* grape berries. LM19 immunolabelling of non-esterified homogalacturonan in thin sections of grape berries. **A** Section of a Control berry (0 h). Labelling is indicated by bright red fluorescence in the pericarp and mesocarp. **B** Control berry at 3 h. **C** NAA-treated berry at 3 h. **D** Control berry at 24 h. **E** NAA-treated berry at 24 h. ep, epidermis, pe, pericarp. Bar = 150 µm

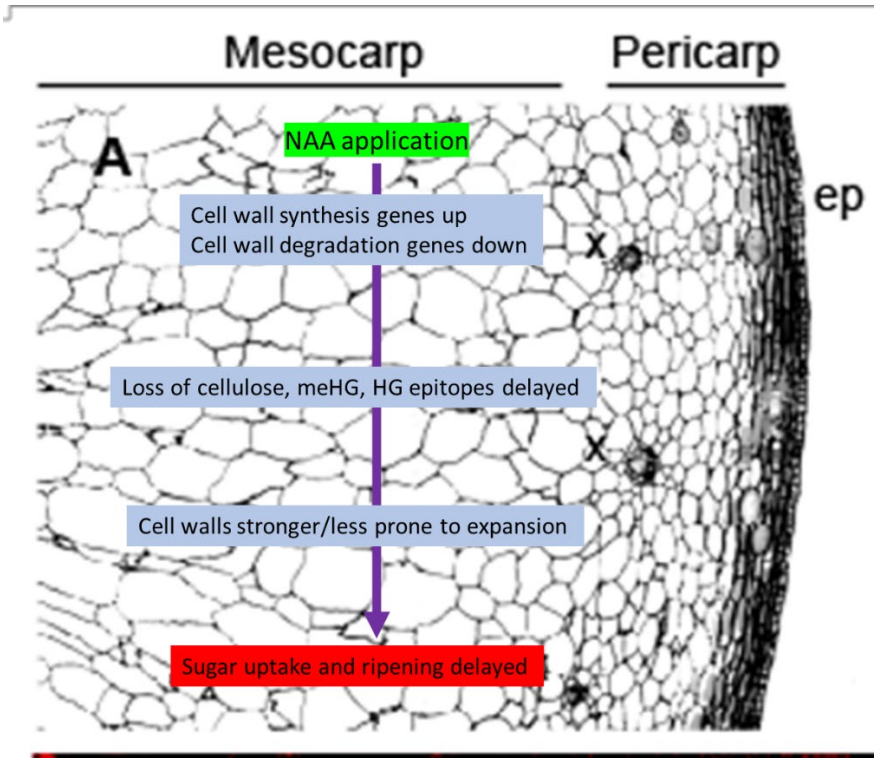
The changes observed in the expression of pectin modifying genes (data not shown) and in the differences in the level of homogalacturonan epitopes (Figures 33, 34 and data not shown) are consistent with NAA treatment delaying the pectin modifications associated with ripening. In grapes the consensus is that the degree of pectin methyl-esterification decreases during grape ripening (Barnavon *et al.,* 2001; Nunan *et al.,* 1998) and that pectin is depolymerised and decreases in amount during ripening in mesocarp and/or

skin tissue (Fasoli *et al.,* 2016; Moore *et al.,* 2014; Nunan *et al.,* 1998; Yakushiji *et al.,* 2001). PME gene expression was down-regulated by auxin treatment in this work (data not shown) as has been observed in mature green tomato fruit (Li *et al.,* 2016) and NAA treatment slowed the de-esterification and degradation of pectin (Figures 33, 34). The down-regulation of PME expression might reduce the degradation of pectin resulting from PG activity. PME gene expression and enzymic activity has been shown to increase during berry ripening in a number of studies (Barnavon *et al.,* 2001; Deytieux-Belleau *et al.,* 2008; Fasoli *et al.,* 2016; Nunan *et al.,* 2001), in agreement with the decrease in the methyl-esterification of pectic polysaccharides. In addition, the expression of genes encoding PG and pectate lyase enzymes were down-regulated by NAA treatment and that of several putative pectin methylesterase inhibitor genes was up-regulated (data not shown).

6.4.3.12 Specificity of NAA-induced changes in gene expression

This study on the effects of NAA on pre-ripening berries in culture, can be used to interrogate a number of alternatives that could be proposed for the mechanism by which NAA treatment prior to veraison controls gene expression to delay ripening; 1) NAA prevents ripening by maintaining the transcriptome in a stable, pre-veraison state (however, this did not happen as there were clearly considerable changes in genes expression during the course of the experiment (Figure 31)), 2) NAA causes changes in the transcriptome that either reset it to a state found at some stage during the pre veraison period or 3) there were changes to the transcriptome not related to an earlier state but nevertheless result in a ripening delay. Even if these changes are not specific to an earlier stage of development, they are repeatable rather than random as pre-veraison auxin treatment consistently delays ripening (Böttcher *et al.,* 2011a, 2012; Böttcher *et al.,* 2011b; Davies *et al.,* 2015).

Figure 35. Schematic of propsed auxin action. Mesocarp, pericarp and epidermis (ep) are indicated



6.4.4 Conclusions

An examination of the changes caused by NAA in relation to their expression in untreated berries was informative regarding the mechanism that delayed ripening. The response to auxin is not a maintenance of the pre-veraison state or a simple resetting of the developmental clock to a pre-veraison state.

The expression of 1,892 genes was significantly changed compared to the Control during a 48 h time-course where the auxin (NAA) was applied to pre-veraison grape berries. Principal component analysis showed that the Control and auxin-treated samples were most different at 3 h post-treatment when approximately three times more genes were induced than repressed by NAA. There was considerable cross-talk between hormone pathways, particularly between those of auxin and ethylene. Decreased expression of genes encoding putative cell wall catabolic enzymes (including those involved with pectin) and increased expression of putative cellulose synthases indicated that auxins may preserve cell wall structure.

Some of the changes must affect key events that delay ripening and some can be considered as 'noise'. For example, apart from the induction of a homeotic response to NAA, a more general stress response might ensue because of the exposure of the berry to auxin at a developmental stage when auxin levels are usually on the decline. There is also the issue that applying NAA when auxin levels are normally low will cause some genes to respond in an atypical way.

The evidence described in this work supports the proposal that NAA addition stabilised the cell wall, or even increased cell wall strength, and delayed ripening-related changes to cell wall structure. Changes to the cytoskeleton that may relate to cell wall saccharide transport and deposition may also be involved in this process. Delaying cell wall loosening could be the key to the ripening delaying effect of auxins. It has been observed previously that one of the first events that occurs during grape ripening is berry softening (Coombe and Bishop 1980; Huang and Huang 2001). Cell division is almost completely halted by the time veraison occurs (Ojeda *et al.,* 1999) and so the second phase of berry size increase is due to cell expansion when there is a concomitant increase in glucose and fructose accumulation. Huang and Huang (2001) showed that cell wall loosening results in tissue softening that in turn allows sugar accumulation, building berry turgor. This pressure results in the cell walls of the skin tissue loosening, allowing berry volume to continue to increase, driven by sugar importation. Based on this model, an auxin-induced delay in the loosening of berry cell walls would delay ripening (Figure 35). Castellarin *et al.* (2016) showed that mechanical restriction of berry expansion delayed softening and inhibited sugar accumulation. The proposed changes to the cell wall described here may also explain the size 'overshoot' that occurs in NAA-treated berries in the field. It has been previously observed that NAA-treated fruit, delayed in ripening, can be significantly larger at harvest than Control berries (Böttcher *et al.,* 2012; Böttcher *et al.,* 2011b). The delay in ripening, which results in an extension of the pre-veraison period and the observed changes to the expression of genes encoding putative cell wall-related enzymes could result in berries with a higher capacity to expand through the increased synthesis of cell wall components.

The data presented above indicate that the delay resulting from the pre-veraison treatment of berries with auxin could have two components. One is a delay in veraison resulting from a disruption of the ripening programme as defined by changes to the transcriptome (described above), and presumably the metabolome. The second is that if the auxin-treated berries expand to a larger size than the untreated fruit (Böttcher *et al.,* 2012; Böttcher *et al.,* 2011b) then more photosynthate is required for them to achieve the same, desired sugar concentration for winemaking which takes more time and therefore also

contributes to the delay in reaching the fully ripe stage. This has been discussed earlier in this report in relation to NAA-treated berries in the vineyard.

6.5 Testing low range NAA application rates for effectiveness in delaying Cabernet Sauvignon veraison/harvest

6.5.1 Introduction

Residues are always an important issue when treating vines with agrochemical reagents. The concentration of NAA to be used will always be a balance between what is reliably and sufficiently effective in delaying ripening/harvest while keeping residue levels as low as possible. To this end we designed an experiment to test the effectiveness of lower range NAA levels.

Cabernet Sauvignon vines at Padthaway, SA were used for this study**.** Fruit set was poor and there was extensive frost and hail damage. As a consequence, ripening was very irregular within and between bunches (Figure 36).



Figure 36. Control bunches at pre-veraison (left) and veraison (right panel) stages showing frost and hail damage and exhibiting poor set.

6.5.2 Materials and methods

A triplicated randomised block design was used for this study. Cabernet Sauvignon bunches were sprayed to runoff with hand-held sprayers with a solution of wetting agent, 0.1% (v/v) Vitiwet (SST Products, South Melbourne, Australia) containing 1 mL/L of 1 M NaOH. Auxin-treated bunches were sprayed to runoff with the same solution but containing different concentrations of NAA (Gibco BRL Life Technologies, Grand Island, USA): 15, 25, 35, 50 mg/L of NAA, taken up in 1 mL of 1 M Na OH. Berries were sampled at 14, 28 and 42 days after treatment and Brix measured by refractometer. Harvest samples were taken at 42 days post treatment frozen and stored at -80^{o}C. Laboratory-scale wines were made and NAA residues were measured as described in the Method section.

6.5.3 Results and Discussion

At 14 days post-treatment there was a significant delay in Brix in all NAA-treated berries compared to the Control berries. The trend was for the fruit treated with the highest NAA concentration (50 mg/L) to be delayed the most (Figure 37). No difference was detected at 28 days post-treatment, which was surprising, however this could have been due to a problem with sampling. At 42 days post-treatment the Brix for all except the 15 mg/L NAA treated fruit was significantly lower than that for the Control fruit. This shows that despite the state of the fruit due to the hail damage and the poor set, even the lowest NAA concentration worked to some extent at two weeks post-treatment and all but 25 mg/L at 42 days post-treatment. Despite the 25, 35, 50 mg/L NAA treatments all having a significant effect on TSS accumulation (Figure 37) the delay was less than expected from previous experience even for the highest NAA concentration. The extent of damage to fruit and the asynchronous development of the fruit may have affected the developmental process and our judgement of veraison may also have been not as precise as hoped which we know can affect the degree of ripening achieved.

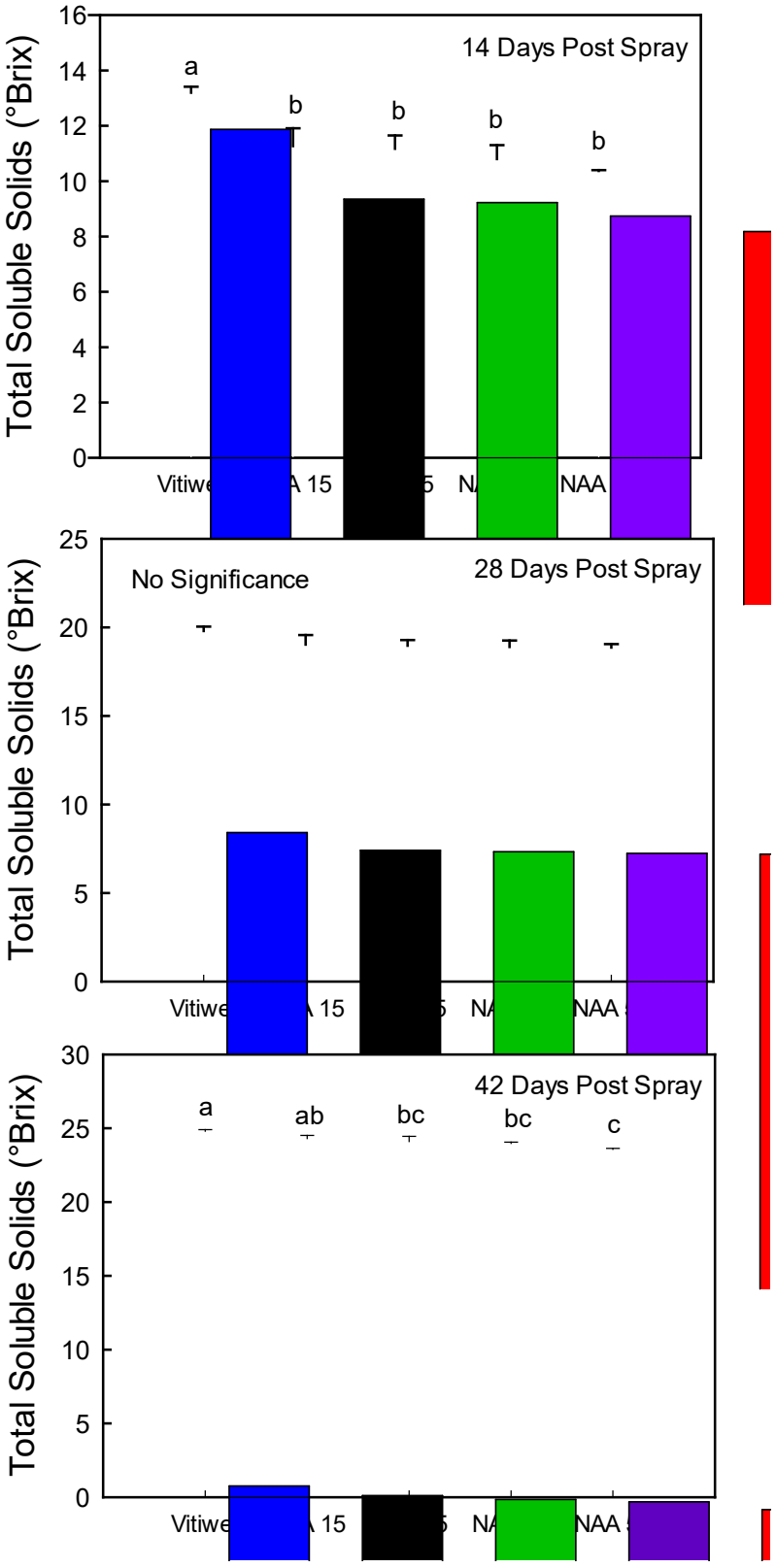


Figure 37. TSS (°Brix) at 14, 28 and 42 post spray with 0 ('Vitiwet'), 15, 25, 35, and 50 mg/L NAA. Lettering denotes significant differences as defined by ANOVA ($P < 0.05$) followed by Duncan's post hoc test.

At harvest, six weeks after treatment, it was observed that both the berries and the rachis of the NAA-treated fruit were in better 'condition' than the untreated fruit. We did not quantify the difference, but the effect can be seen in the photograph of two bunches that appeared healthy and undamaged earlier in the season (Figure 38). The NAA-treated fruit seemed more intact and there was less berry shrivel. This could increase both berry quality and yield. The NAA treatment also seemed to preserve the function of the rachis better than the Control treatment. The treated rachides were green and healthier looking, in a number of Control bunches (not shown) there was widespread browning and death of some parts of the bunches. It suggests that there may be a role for auxins in reducing berry loss due to bunch stem necrosis which is a significant issue, in the South East of South Australia for example, that causes crop loss and reduced berry quality.



Figure 38. Photos of typical bunches taken at 42 days post treatment. Left Control, right NAA-treated bunch.

NAA residues were measured in berries from all five treatments. Residue concentration was highest in the fruit treated with 50 mg/L NAA but even so, was below 0.005 ppm, which would be below international MRLs, which are generally set at 0.06 – 0.1 ppm (Figure 39).

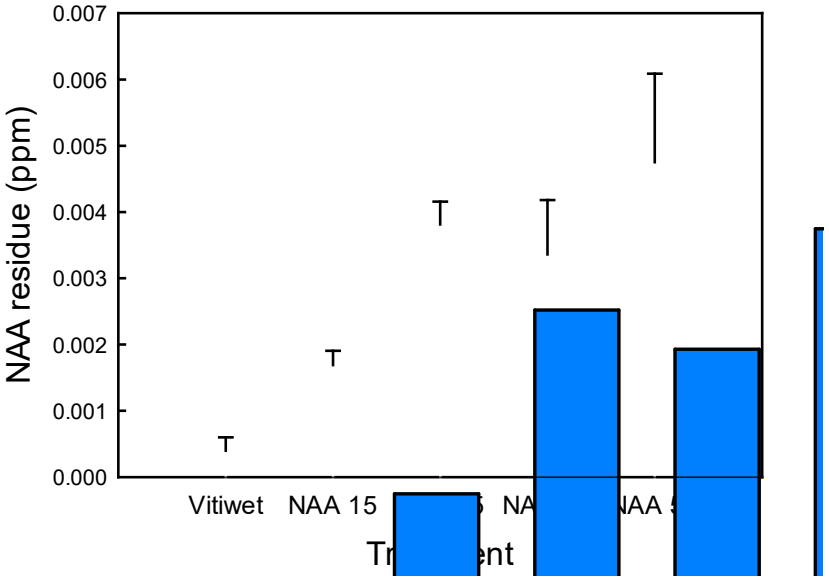


Figure 39. NAA residues in wine made from grapes (harvest 42 days after treatment) treated with 0, 15, 25, 35 and 50 mg/L NAA

6.6 Ripening delay resulting from NAA application is NAA concentration dependent

6.6.1 Introduction

We have seen that higher concentrations may be more effective in delaying ripening/harvest, however, the effect of the concentration of NAA applied to bunches on the extent of the delay and any changes to berry size and composition has yet to be fully analysed. Low levels may be desirable, but they may not be able to induce a practically useful and reliable delay in harvest. Equally, overdoing the application rates may not further increase the delay and may have negative effects on berry and wine composition and residue level. In this trial we sought to test the effect of the application of a wide range of NAA concentrations on ripening delay and berry residue levels.

6.6.2 Materials and methods

An experiment was conducted to determine the effect of applying different concentrations of NAA on berry phenology and composition and wine aroma compound composition using *Vitis vinifera* L. cv. Cabernet Sauvignon vines. Drip-irrigated, double cordon vines were located in a commercial vineyard at Padthaway, South Australia. The trial was of a randomised, triplicated design. Two hundred and fifty bunches were used for each treatment; the treatments were on separate vines. Control bunches were sprayed to runoff with a solution of wetting agent, 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton, Australia) containing 1 mL/L of 1M NaOH. Auxin-treated bunches were sprayed to runoff with the same solution but containing different concentrations of NAA, the NAA was taken up in 1 mL of 1M Na OH. Four different concentrations were used 25, 100, 175, 250 mg/L NAA (Gibco BRL Life Technologies, Grand Island, USA), these treatments will be referred to as 'N25', 'N100', 'N175' and 'N250' respectively. Treatments were carried out 10 days prior to veraison. Veraison was determined as the sampling time-point where Control fruit showed an increase in TSS. At harvest approximately 25 kg of fruit was harvested for conventional small-scale winemaking (WIC Winemaking Services) and for smaller scale laboratory ferments. A subsample of 70 berries per replicate was taken for berry weight determination and TSS measurement. The harvest dates were, Control (78 dps), N25 (88 dps), N100 (95 dps), N175 and N250 (99 dps). To test residue levels an additional 60 berries /replicate were sampled randomly, washed with distilled water and stored at -40^{o}C until analysed as in Methods.

6.6.3 Results and discussion

6.6.3.1 The effect of different NAA concentrations on berry weight and TSS accumulation

All four of the concentrations of NAA applied affected berry weight increase (Table 22). The mean harvest weight for Control fruit was 0.79 g/berry at 79 days post spray (dps) while the harvest weight for berries treated with NAA was 0.88, 0.89, 0.97 and 1.06 g/berry at 88, 95, 99 and 99 dps for N25, N100, N175 and N250 respectively. The NAA-treated berries reached a higher mean weight than the Control fruit and the increase in weight correlated with the increase in NAA concentration applied. However, the only statistically significant increase occurred with the 250 mg/L treatment, and this was 134% of the Control berry weight.

Table 22. Berry weight, Brix and stored photosynthate content for Control and NAA-treated berries at harvest. Letters represent statistically significant groupings as determined by ANOVA analysis ($P < 0.05$) followed by Tukey's post hoc test.

	Berry weight (g)	°Brix	Brix X weight
Control	0.79b	27.13	21.43b
N25	0.88ab	27.23	23.96ab
N100	0.89ab	27.73	24.68ab
N175	0.97ab	27.27	26.45ab
N250	1.06a	26.93	28.55a

As for berry weight, the delay in TSS accumulation was also concentration dependent except that for the two highest NAA concentrations (N175 and N250) the delay was almost identical (Figure 40). This suggests that there was a saturation effect when applying more NAA did not significantly increase the delay in TSS accumulation. The Brix at harvest was not statistically different between the Control and NAA-treatments (Table 22) and the harvest was 8, 16, 20 and 20 days after the harvest of the Control for N25, N100, N175 and N250 respectively. Even the lowest NAA concentration produced an acceptable delay in harvest timing.

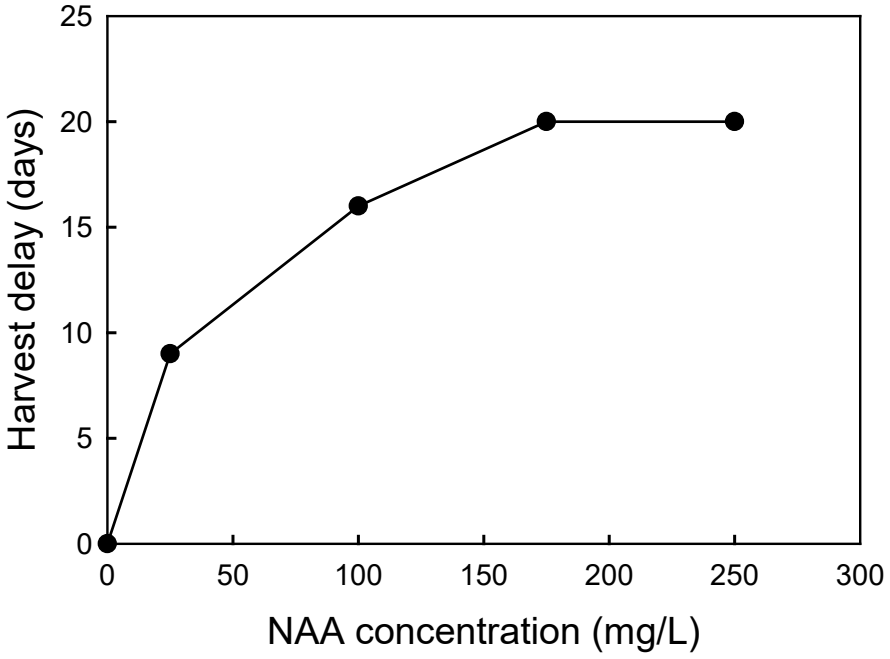


Figure 40. Delay in reaching harvest relative to NAA concentration of treatment

To calculate the total amount of carbon stored by the berries for each of the different treatments the mean weight was multiplied by the mean TSS concentration (Table 22). Carbon storage was higher at harvest for the NAA-treated fruit compared to the Control, again the increase seemed NAA concentration dependent as the NAA250 treatment stored the most carbon and was the only treatment showing a statistically significant increase.

The delaying effect of even the lowest NAA concentration used can also be seen visually. Three weeks after the NAA treatment all of the NAA-treated fruit was delayed as demonstrated by delayed skin colour accumulation (Figure 41).

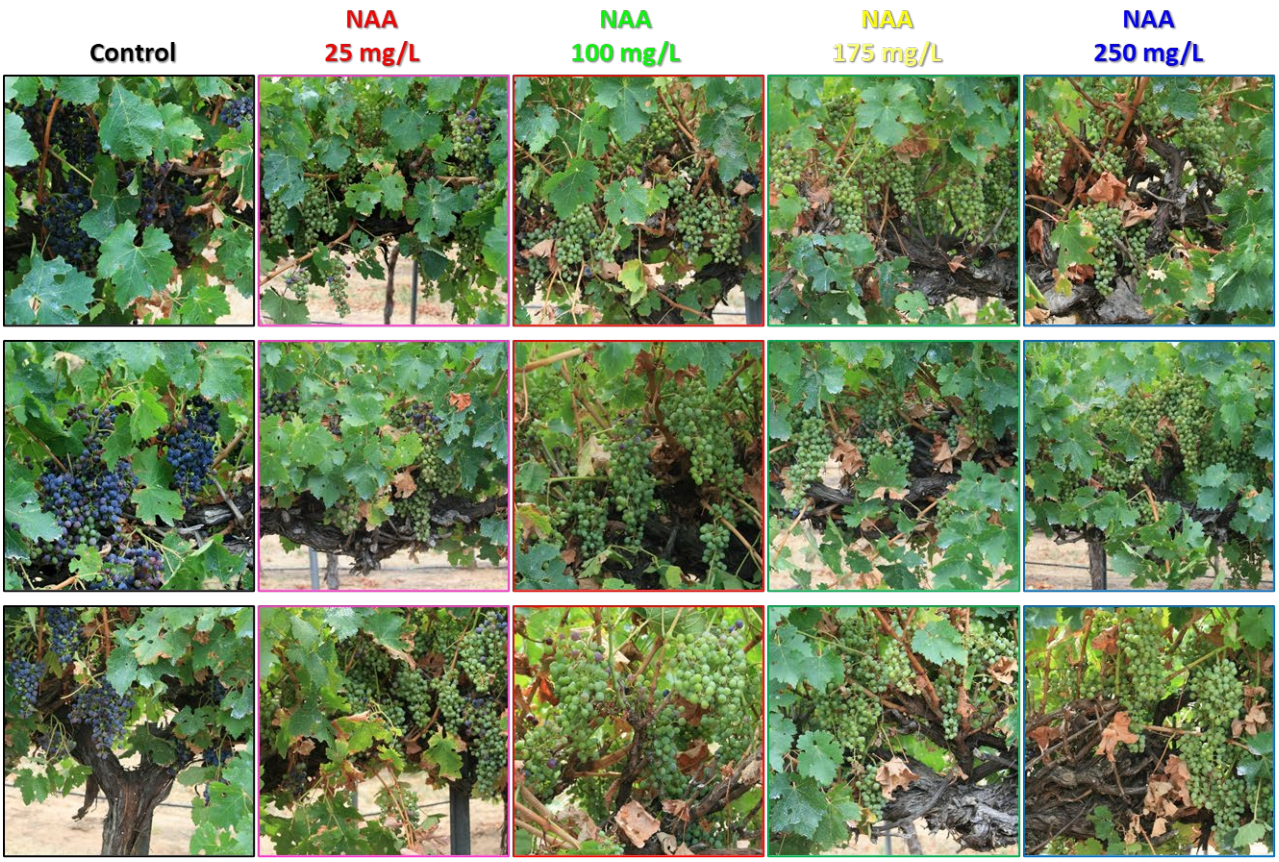


Figure 41. Photographs of typical bunches taken for Control and NAA-treated fruit 21 days after treatment. Examples of all three replicates are shown.

The methoxypyrazine, 3-isobutyl-2-methoxypyrazine (IBMP) is an important contributor to wine sensory properties. It is highly odour active and at lower concentrations may add complexity to wine but at higher levels, in Cabernet Sauvignon wines for example, it can impart an undesirable 'green' character. At harvest the concentration of IBMP was lower in NAA-treated berries, particularly in fruit treated with higher NAA concentrations (Figure 42).

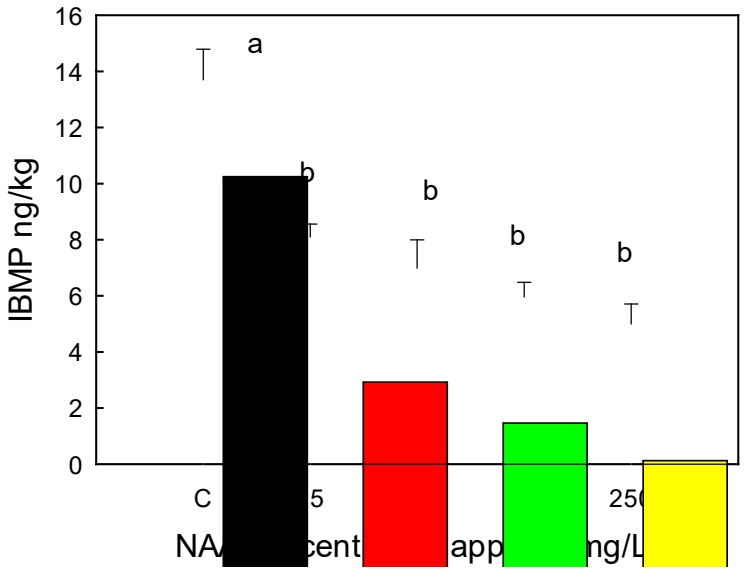


Figure 42. The concentration of IBMP (ng/kg) in Cabernet Sauvignon berries at harvest after treatment with increasing concentrations of NAA. Letters represent statistically significant groupings as determined by ANOVA analysis ($P < 0.05$) followed by Tukey's post hoc test.

The fruit from the five different NAA concentration treatments was harvested for winemaking when they reached, as near as possible, the same TSS concentration. Analysis of the juice at crushing (Table 23) shows that there was no significant difference in Brix at harvest. The pH was lower in the juice from N175 and N250 grapes while TA 8.2 and malic acid concentration were higher in juice from NAA-treated fruit, with the exception of malic acid in the N100 juice having a similar to the concentration to the Control. YAN concentration was significantly lower in the juice from NAA-treated fruit compared to the Control, with the exception of the juice from N100 berries. In previous studies, YAN has been higher in NAA-treated fruit, which shows that there is some inconsistency in some berry property measurements.

Table 23. Measurements of TSS (˚Brix), pH, TA (g/L), malic acid (g/L) and YAN (mg/L) in berries at crushing. Letters represent statistically significant groupings as determined by ANOVA analysis ($P < 0.05$) followed by Tukey's post hoc test.

	˚Brix	pH	TA 8.2	Malic acid	YAN
Control	26.9	3.90a	2.97b	1.32b	182a
N25	26.7	3.80abc	3.67a	1.87a	154b
N100	27.4	3.85ab	3.83a	1.62ab	178a
N175	27.0	3.75bc	3.80a	1.84a	162b
N250	26.9	3.72c	3.87a	1.78a	156b

The wine volatile data are still being analysed, but a preliminary analysis shows that there are some changes in the concentration of some of the 78 volatile compounds that are clearly identified in the wine from Control and N25 fruit. The initial analysis indicates that the differences are small and that the delay has not dramatically changed berry and wine composition. In the N100 wine there is a general trend for the volatiles in wine from treated fruit to be lower in concentrations, but the profiles of N25 and N100 are nevertheless very similar. These small changes in volatiles could be due to the pre-veraison period being extended and because the delaying of ripening tends to push ripening into a cooler time of the year. This may be a good outcome as cooler region wines seem have more complex flavour/aroma profiles. A clear change is seen in the volatile profiles of N175 and N250 wines, where there is an increase in the concentration of most of the identified volatiles. This may not be a desirable effect as the aroma profile may become unbalanced, and some of the compounds that increased in concentration may be deleterious at higher levels. It may be that the higher NAA concentrations stress the berries and the increase in volatiles is a response to perceived injury. Luckily, NAA concentrations of 100 mg/L or less are all that is required to provide a delay in harvest that is of a suitable duration to ease harvesting and winery scheduling problems resulting from climate change-induced seasonal compression.

6.7 Testing for possible interactions between NAA and two commonly used fungicides and an insecticide that might impact on NAA efficacy in delaying ripening

6.7.1 Introduction

If NAA applications could be applied in the same mix as fungicides and insecticides, it would save an additional pass with spray equipment and therefore reduce fuel, machinery and labour costs, as well as reducing soil compaction between rows. It is possible that despite its relative stability, agents like wettable sulfur could interact and impact the effectiveness of NAA in delaying ripening.

6.7.2 Materials and methods

Shiraz vines located at Willunga, SA, were used for this experiment. A randomised, triplicated design was used (Figure 43). Flowering was 14 Nov 2017, the spray treatment was conducted on 2 Jan 2018, one week before veraison. Sixteen bunches per replicate were sprayed to runoff with the following solutions:

- Control (0.1% Chemwet, 1 mL/L 1M NaOH)
- NAA (100 mg/L NAA, taken up in 1 mL/L 1M NaOH, 0.1% Chemwet)
- Yates Nature's Way Caterpillar Killer (Dipel (1 g/L = 8.64 million units of *Bacillus thuringiensis*) + NAA 0.1% Chemwet, 1 mL/L 1M NaOH))
- Amgrow Mancozeb (2 g/L = 1.5 g active ingredient) + NAA, 0.1% Chemwet, 1 mL/L 1M NaOH))
- Microfine wettable sulfur (2 g/L = 1.6 g active ingredient) + NAA, 0.1% Chemwet, 1 mL/L 1M NaOH))

Berries were sampled (80-90 berries per replicate) 2, 7, 14, 21 days after spraying. At harvest (29 days post treatment) all remaining Control and treated fruit was harvested. Brix measurements were carried out using a digital refractometer.

Wettable sulfur+NAA	Control	NAA
Mancozeb+NAA	wettable sulfur+NAA	Dipel+NAA
Dipel+NAA	NAA	Control
NAA	Dipel+NAA	Mancozeb+NAA
Control	Mancozeb+NAA	wettable sulfur+NAA
Rep 3	**Rep 2**	**Rep 1**

Figure 43. Design of trial showing randomised distribution of treatments over three rows

6.7.3 Results and discussion

The presence of Dipel, Mancozeb and wettable sulfur did not appear to affect the NAA-induced delay of ripening (Figure 44). All treatments containing NAA, with or without added fungicide or pesticide, delayed Brix accumulation to the same extent during ripening.

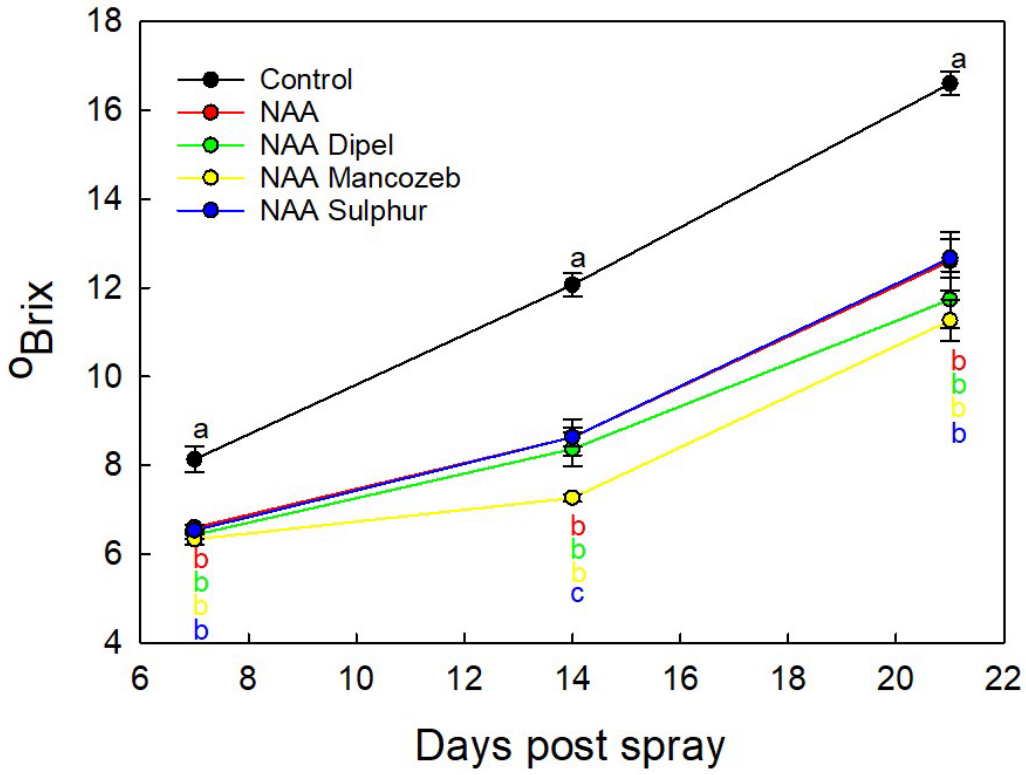


Figure 44. TSS (°Brix) of berries treated with NAA, with and without fungicides and insecticide. Measured at 7, 14 and 21 days post treatment. Lettering denotes significant differences as defined by ANOVA ($P < 0.05$) followed by Duncan's post hoc test.

The delay in ripening persisted to harvest where there was a statistcally lower Brix in all treatmenst containing NAA compared to the Control (Figure 45). There was no significant difference between the Brix levels of berries treated with NAA alone or when treated with NAA in combination with either fungicides or the insecticide. Although there are numerous other fungicides that could be tested, these preliminary outcomes are encouraging and are in line with NAAs relative inertness.

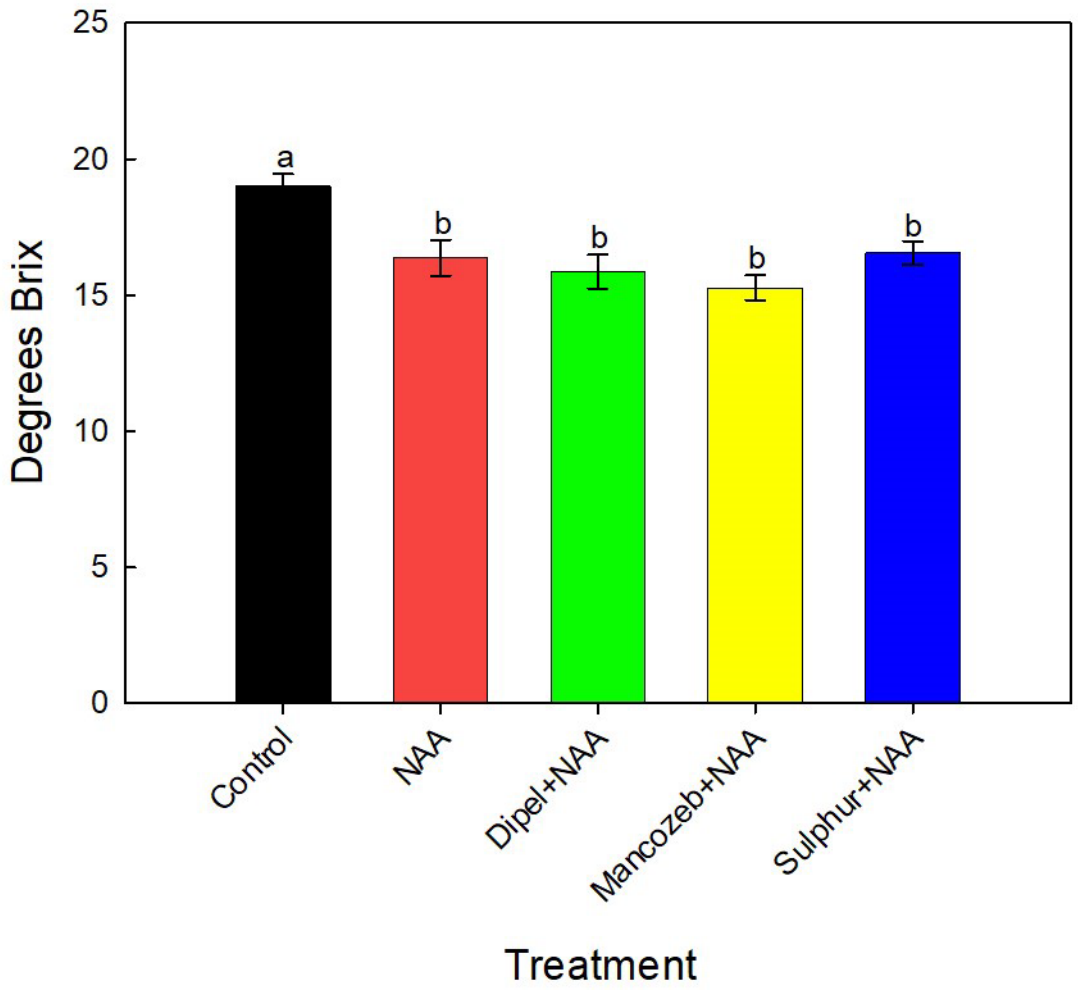


Figure 45. TSS (°Brix) of berries treated with NAA, with and without fungicides and insecticide. Measured at harvest, 29 days post treatment. Lettering denotes significant differences as defined by ANOVA ($P < 0.05$) followed by Duncan's post hoc test.

Berry weights were not significantly different at harvest (data not shown). As expected, because the NAA-treated fruit was less ripe at harvest (the fruit was harvested on the same day for Control and NAA-treated samples) the anthocyanin levels were lower in NAA-treated fruit and the total acid concentration was higher due to higher levels of malic acid (data not shown).

In summary, the presence of the additional reagents did not affect the ripening-delaying properties of NAA contained in the same spray solution.

6.8 Testing for possible interactions between NAA and four wetting agents with different chemistry - impact on NAA efficacy in delaying ripening and NAA residue levels

6.8.1 Introduction

This experiment was designed to further test parameters that might impact on the efficacy of NAA application by spraying. Adjuncts are often used in commercial formulations of agrochemicals. Sometimes these compounds are designed to 'stick' the active ingredient to the surface of the plant, or to aid in penetration. In many cases wetting agents are used to ensure good coverage and entry of the reagent into the plant. As the berry surface has a waxy coat, it is sensible to add wetting agents (surfactants) to the spray mix. In this experiment four commercial wetting agents with different chemistries were evaluated in conjunction with NAA.

6.8.2 Materials and Methods

Shiraz vines located at Willunga, SA, were used for this experiment. A randomised, triplicated design was used (Figure 46). Flowering was 14 Nov 2017, the spray treatment was conducted on 2 Jan 2018, one week before veraison. Sixteen bunches per replicate were sprayed to runoff with the following solutions:

- Control (Chemwet, NaOH)
- no wetting agent Control (100 mg/L NAA, taken up in 1 mL/L 1M NaOH)
- Viti-Wet at 0.01% (100 µL/L) + NAA
- Silwet L-77 at 0.05% (500 µL/L) + NAA
- Banjo at 0.5-1% (5-10 mL/L) + NAA
- Chemwet1000 at 0.1% (1 mL/L) + NAA
- 100 mg/L NAA, taken up in 1 mL/L 1M NaOH added to each

Berries were sampled (80-90 berries per replicate) 2, 7, 14, 21 days after spraying. At harvest (29 days post treatment) all remaining Control and treated fruit was sampled. Brix measurements were carried out using a digital refractometer.

NAA residues were measured by LC-ESI-MS/MS as described in Method section.

Banjo	Control	Silwet
Silwet	Chemwet	NAA no WA
Viti-Wet	Banjo	Chemwet
Chemwet	NAA no WA	Control
NAA no WA	Silwet	Viti-Wet
Control	Viti-Wet	Banjo
Rep 3	Rep 2	Rep 1

Figure 46. Design of trial showing random distribution of 'wetting agent' treatments over three rows

6.8.3 Results and Discussion

At harvest, the only treatments that were significantly different to the Control were those where the wetting agents Banjo or Viti-Wet were added (Figure 47). At no stage were the berries treated with Silwet combined with NAA significantly different in Brix from the Control. The NAA treatment without wetting agent and the treatment of NAA combined with Chemwet were effective in delaying ripening at 7, 14 and 21 days but there was no significant difference at harvest. The treatments with NAA combined with either Banjo or Viti-Wet were effective in delaying ripening at all time-points after treatment. These results are suggestive, but we must be careful in their interpretation with regard to treatment in a commercial setting. The scale of the trial was quite small and replication in a larger trial using commercial equipment would improve confidence in the outcomes. Obviously, NAA still has some effect even without a wetting agent. There is also an indication that some wetting agents may be more effective than others. The best way to test this would be as described above i.e. completing a trial with more vines/replicates, preferably in a commercial setting testing NAA concentration effects at the same time. It is difficult to do small-scale studies to determine very small effects.

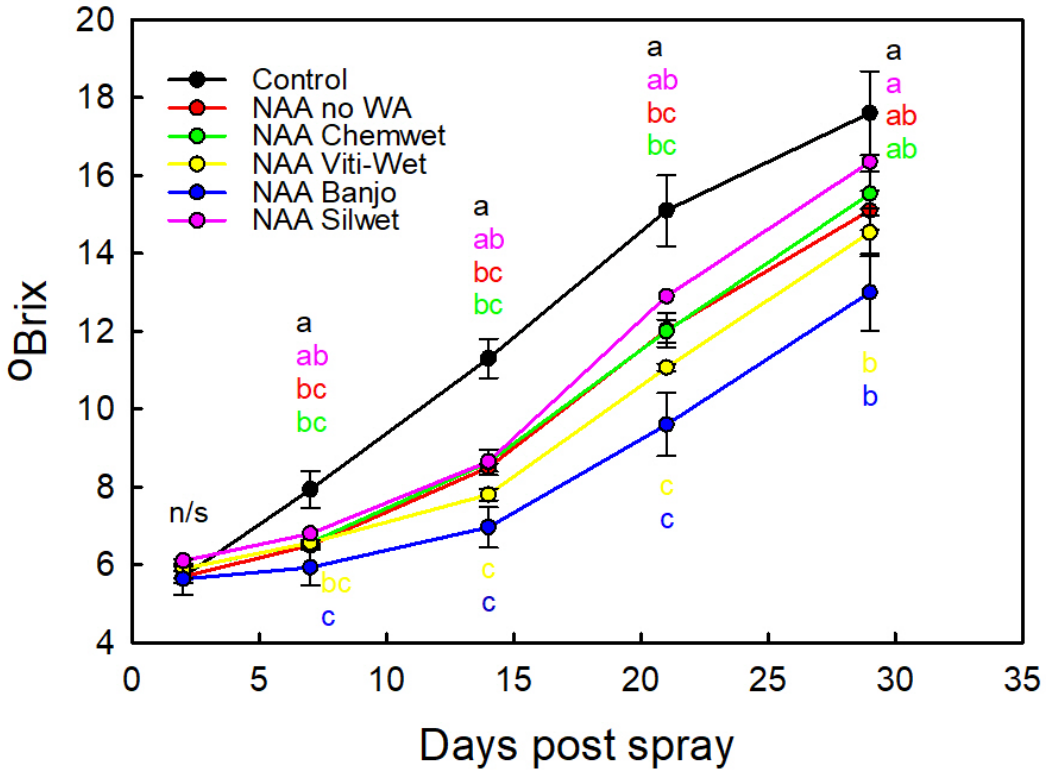


Figure 47. TSS (°Brix) of berries treated with NAA with and without a range of wetting agents. Lettering denotes significant differences as defined by ANOVA ($P < 0.05$) followed by Duncan's post hoc test.

In terms of the effect of wetting agent on the NAA residue in/on berries, there appeared to be no significant difference at harvest between the different agents (Figure 48). This suggests that wetting agents may have some effect on ripening but are not likely to influence the concentration of NAA residues. Again, this could be tested in a larger trial.

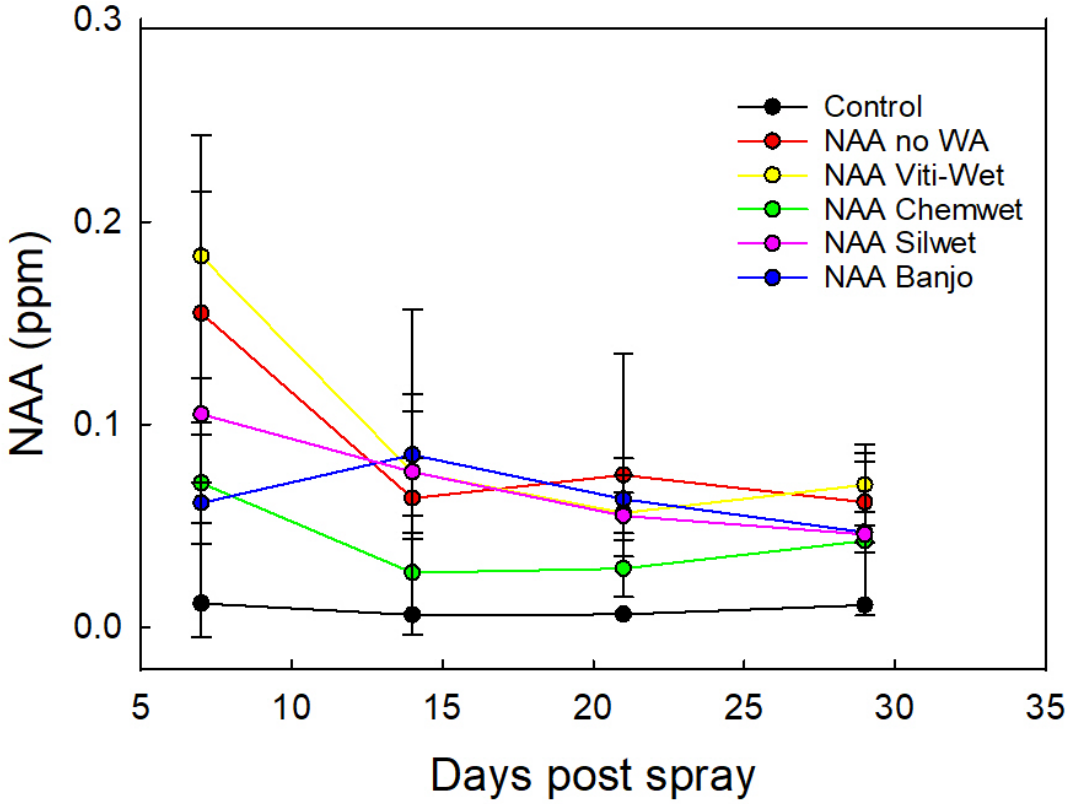


Figure 48. NAA residue ppm (mg/L) of berries treated with NAA with and without a range of wetting agents. Lettering denotes significant differences as defined by ANOVA ($P < 0.05$) followed by Duncan's post hoc test.

6.9 Testing the effect of complete vine spray vs bunch-targeted sprays on vine health and on the length of delay in ripening

6.9.1 Introduction

This experiment was designed to test NAA and Ethrel treatments of whole vines compared to bunches alone to determine whether:

- applying the PGRs to whole vines, including bunches, was more effective than just applying reagents to bunches
- the application to leaves causes damage to the leaves/vine structure
- Ethrel might be a useful alternative or adjunct to using NAA to delay ripening/harvest

6.9.2 Materials and methods

Shiraz vines located at Willunga, SA, were used for this experiment. The trial was of a randomised, triplicated design (see Figure 49 for the layout and flagging tape colours/pattern used). For each whole vine treatment replicate, a backpack sprayer was used to cover all shoots, leaves and bunches. For the bunch-only treatments, six bunches were treated (three per side of each row) with hand sprayers. Three treatments were applied: Control 0.01% Vitiwet in water, NAA20 diluted to NAA 100 mg/L in Control solution, Ethrel 600 µL/L in Control solution. Three unsprayed vines were left between each treatment. The Ethrel treatment was conducted on 22 December 2020. This was earlier than for NAA as our previous work has shown that Ethrel can delay ripening at this stage of development, but later applications may advance ripening. NAA was applied a week later; both applications were before the onset of ripening.

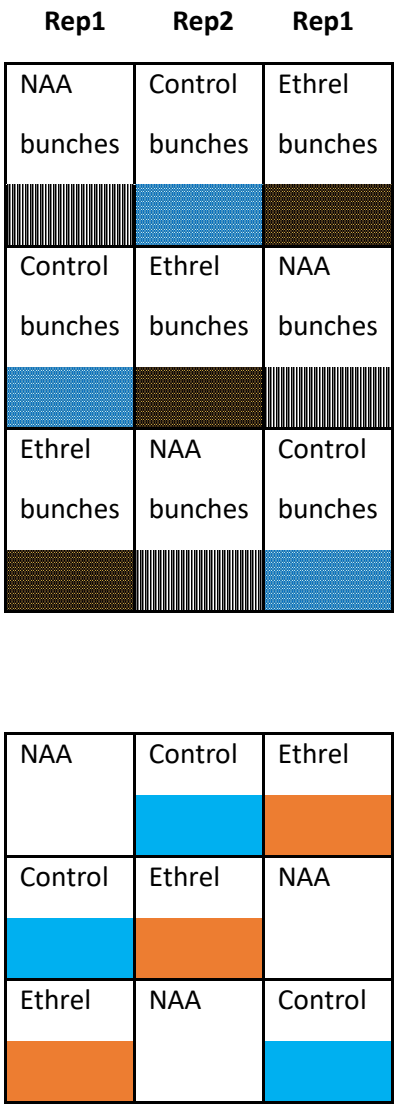


Figure 49. Experimental layout showing arrangement of replicates for whole vine treatments (lower panel) and 'bunch' treatments (upper panel)

On 20 Jan, 2 and 17 Feb, 60 berries per replicate (10 berries from each of the six bunches for the 'bunch' treatments) were taken and analysed for weight and Brix and for other parameters by OenoFoss analysis.

6.9.3 Results and Discussion

Control and NAA-treated vines were very similar in appearance with no obvious effects on leaf colour or health (Figure 50). In contrast Ethrel treatment appeared to hasten the senescence of the treated leaves with obvious signs of yellowing, reddening and death. It seemed to be more the older leaves that were affected and there is insufficient information to assert that this treatment neccesarly decreased overall vine health in the medium to longer term.



NAA-treated **Ethrel-treated**

Figure 50. Appearance of NAA-and Ethrel-treated Shiraz vines. Photos taken 11 Jan, 20 days after Ethrel treatment and 13 days after NAA treatment.

In general the biochemical parameters measured were similar for each treatment whether the entire vine was treated or just the six bunches per vine were treated. There was some variation in some cases, probably because the number of bunches treated was quite low in the case of the bunch alone treatments. As usual the berry weight measurements were the most variable. At the first timepoint the NAA-treated bunches had berries that were lighter than any of the others (Figure 51a). There was no significant difference at the second timepoint (Figure 51b) but at the last time-point the berries from the whole vine NAA treatment had significantly higher mass than fruit from any of the other whole vine or bunch treatments (Figure 51c).

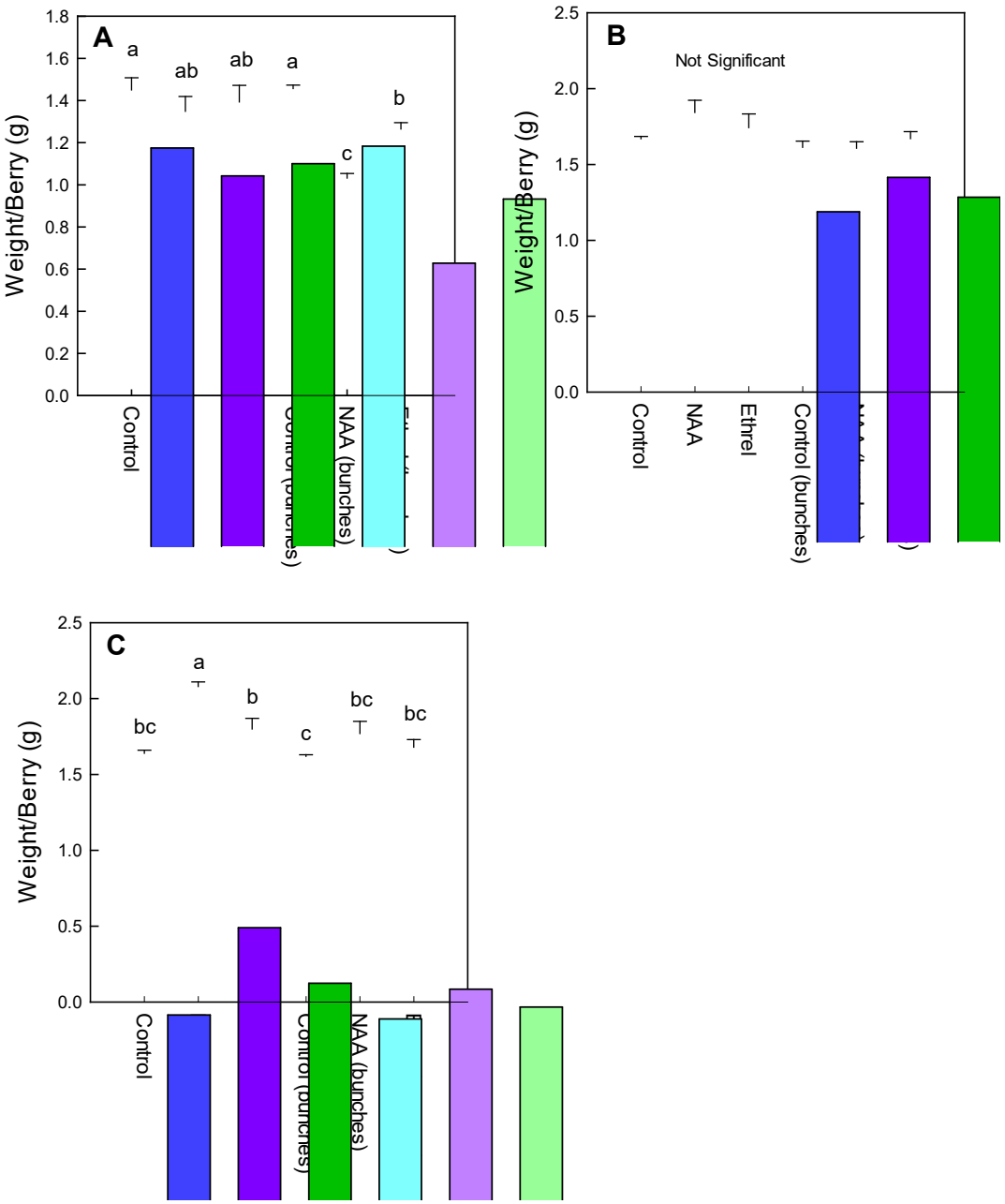


Figure 51. Berry weights for NAA and Ethrel vine and bunch treatments. A) All sampled on 20/01/2021, 22 days post spray for Control and NAA, 29 days post spray for Ethrel B) All sampled on 2/02/2021, 35 days post spray for Control and NAA, 45 days post spray for Ethrel C) All sampled on 17/02/2021, 50 days post spray for Control and NAA, 57 days post spray for Ethrel. Lettering denotes significant differences as defined by ANOVA($P < 0.05$) followed by Duncan's post hoc test.

There was a significant delay in TSS accumulation at all time-points for both NAA and Ethrel treatments (bunch and whole vine). The delay was greater for NAA treatments compared to Ethrel treatments (Figure 52).

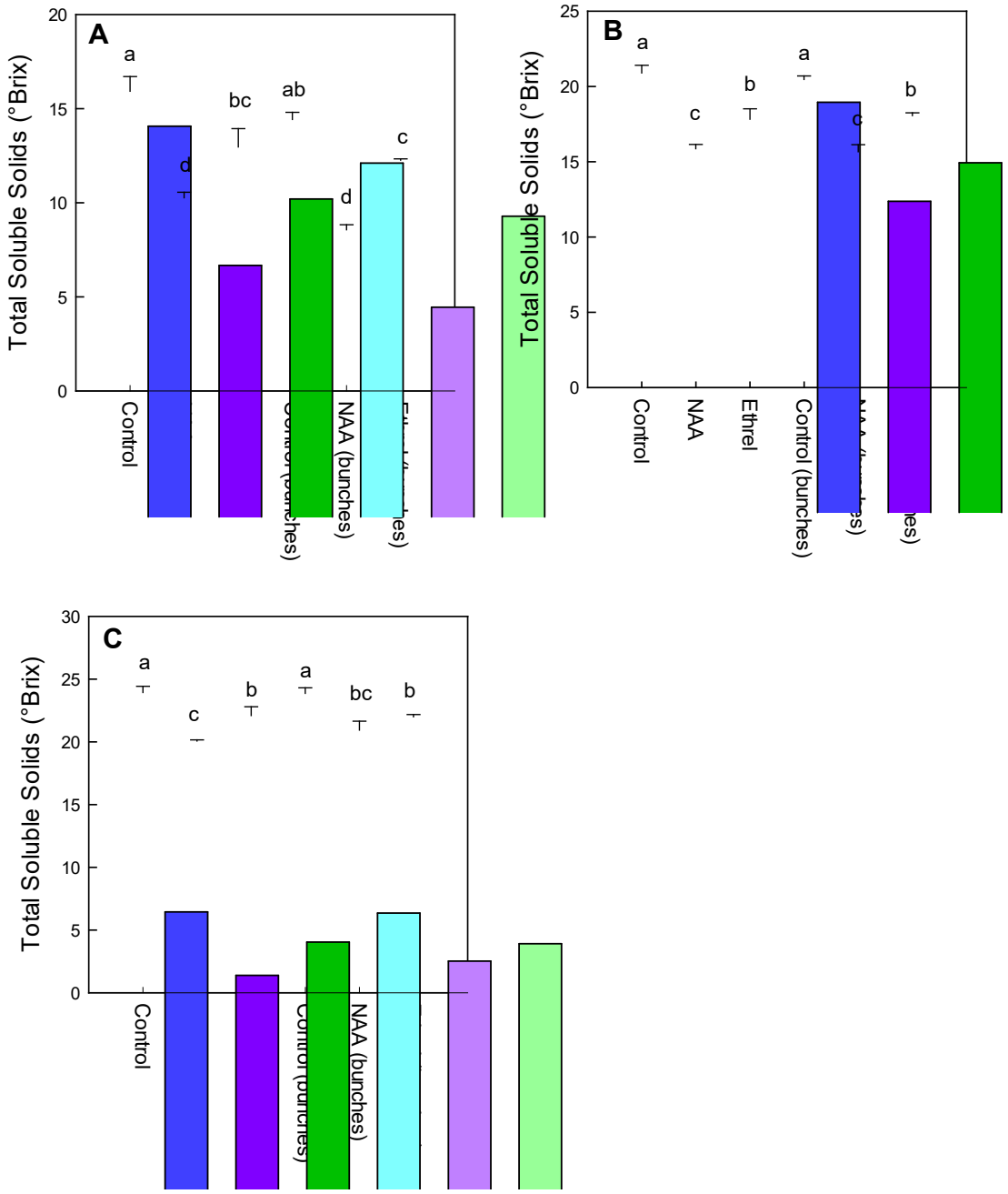


Figure 52. Berry TSS for Control, NAA and Ethrel vine and bunch treatments. A) All sampled on 20/01/2021, 22 days post spray for Control and NAA, 29 days post spray for Ethrel B) All sampled on 2/02/2021, 35 days post spray for Control and NAA, 45 days post spray for Ethrel C) All sampled on 17/02/2021, 50 days post spray for Control and NAA, 57 days post spray for Ethrel. Lettering denotes significant differences as defined by ANOVA($P < 0.05$) followed by Duncan's post hoc test.

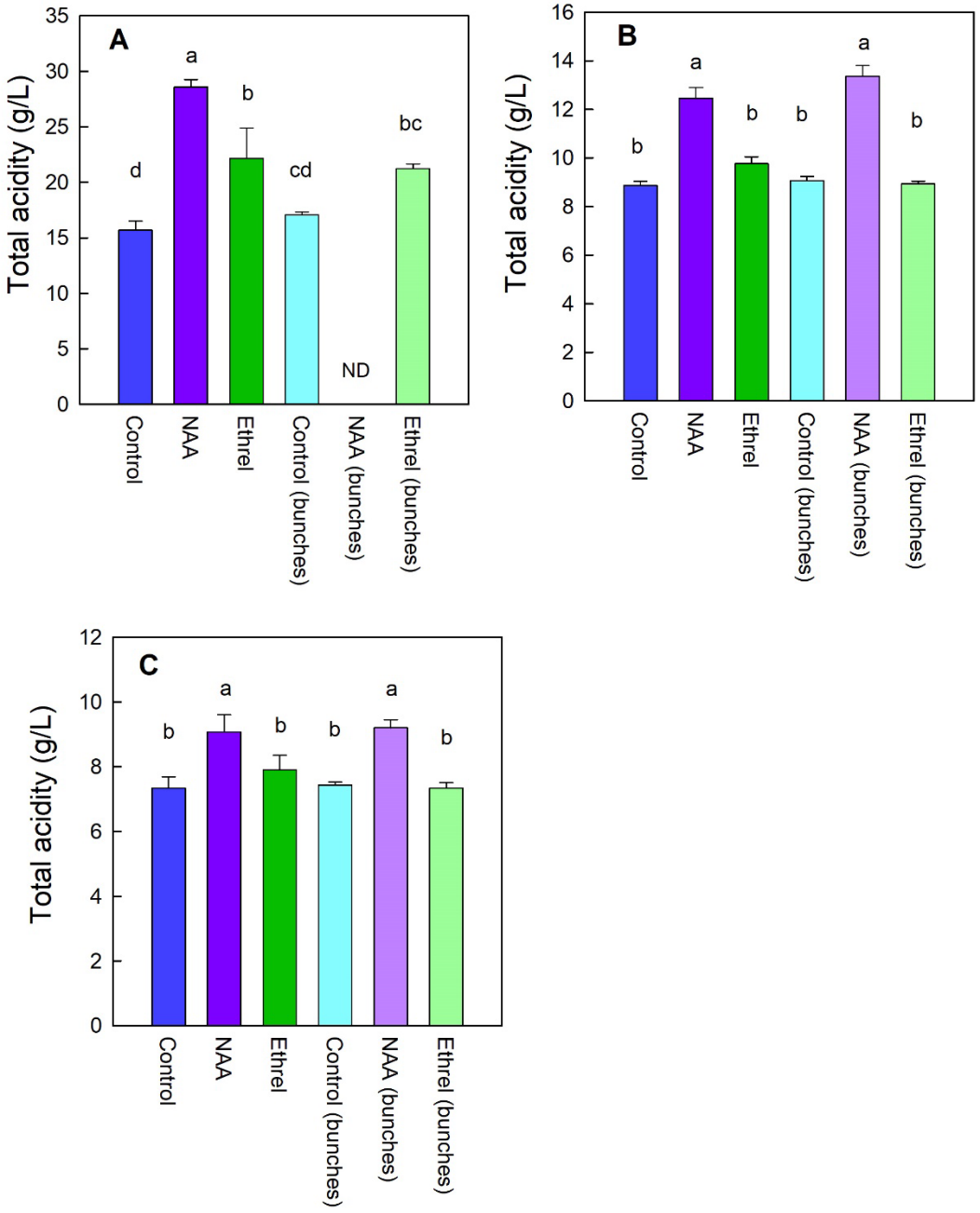


Figure 53. Berry total acidity for Control, NAA and Ethrel vine and bunch treatments A) All sampled on 20/01/2021, 22 days post spray for Control and NAA, 29 days post spray for Ethrel B) All sampled on 2/02/2021, 35 days post spray for Control and NAA, 45 days post spray for Ethrel C) All sampled on 17/02/2021, 50 days post spray for Control and NAA, 57 days post spray for Ethrel. ND = concentration could not be determined. Lettering denotes significant differences as defined by ANOVA($P < 0.05$) followed by Duncan's post hoc test.

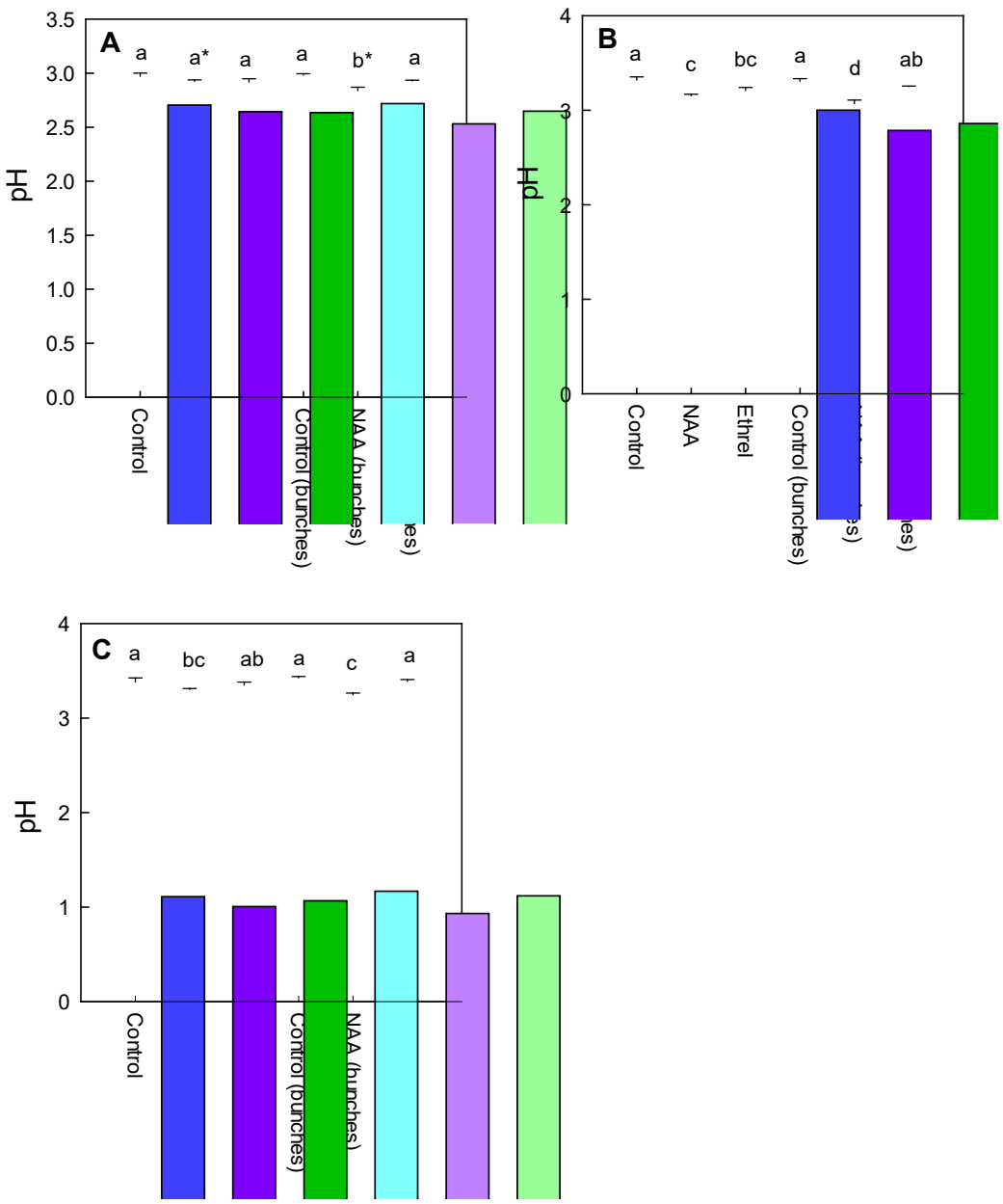


Figure 54. Berry pH for Control, NAA and Ethrel vine and bunch treatments. A) All sampled on 20/01/2021, 22 days post spray for Control and NAA, 29 days post spray for Ethrel B) All sampled on 2/02/2021, 35 days post spray for Control and NAA, 45 days post spray for Ethrel C) All sampled on 17/02/2021, 50 days post spray for Control and NAA, 57 days post spray for Ethrel. ND = concentration could not be determined. Lettering denotes significant differences as defined by ANOVA($P < 0.05$) followed by Duncan's post hoc test.

In line with the total acidity being higher in some of the Ethrel and NAA-treated fruit, the juice pH was lower in some samples (Figure 54). At the second time-point, both NAA treatments had a lower pH while only the whole vine Ethrel-treated sample had a lower pH. At the third time-point only the NAA treatments produced berries with a lower pH.

Both Ethrel and NAA treatments delayed ripening as measured by a delay in the increase in TSS compared to the Control and the delay in the decrease of total acidity with the consequent delay in the increase in pH. Despite both reagents working to delay ripening, the NAA treatment was more effective. It is also the

preferred reagent, as the Ethrel treatment did seem to advance leaf senescence. Our data do not suggest that the bunch-alone treatment was less effective than the entire vine treatment, meaning that spraying of the fruiting zone alone would be adequate in delaying ripening/harvest. Targeting of the fruiting zone should reduce the amount of reagent sprayed in a commercial setting without affecting the efficacy of treatment.

6.10 Determining the location of NAA residue in treated grape berries

6.10.1 Introduction

The issue of NAA residues in grapes and wine following treatment is important when considering the commercial use of this ripening-delaying agent. The location of the residue is of practical interest i.e. whether it is in the berry or on the outer surface of the skin. If it is the latter, then there may be formulations that might not retain NAA on the skin surface for a long period. Modifying formulations might be one way of reducing the residues.

6.10.2 Materials and methods

Shiraz vines located at Willunga, SA, were used for this experiment. Six bunches were treated to runoff with 0.1% Chemwet containing 100 ppm NAA at 15 days prior to veraison (26/12/2018). At 61 days after treatment (25/2/2019), the bunches were harvested, 60 berries were taken from each of three bunches and were frozen in liquid nitrogen and stored at -80^{o}C until assayed (treated with NAA but not washed). Another sample of eighty berries was harvested from each of the remaining three bunches. Sixty berries were washed with 0.1% Chemwet in 80% ethanol followed by a wash in 70% ethanol. These were then dried and then frozen as above. The remaining 60 'washed' berries, 20 from each washed bunch were pooled into a single sample, the wax removed from each berry using paper tissues and then frozen as above. The frozen berries were ground under liquid nitrogen and then 100 mg from each replicate was assayed for NAA concentration as described in Method section. The Brix of the ground powder was measured using a digital refractometer. The average TSS at harvest was 24.3 ^{o}Brix.

6.10.3 Results and Discussion

There was no significant difference in the concentration of NAA in any of the samples (Figure 55). The berries that had been washed and those that had been washed and 'polished' had roughly the same concentration of residues. We might expect that as the NAA is sprayed over the surface of the berry that the concentration in the outer tissues might be higher and that there may be a considerable amount of NAA still on the surface of the skin. However, the results show that extensive washing with detergent and ethanol (a good solvent for NAA) did not reduce the concentration of NAA (Figure 55). It was still possible that the wax on the berry surface may have bound up the residual NAA in a form that could not be effectively washed off. However, when the wax was wiped from the berry surface before analysis there was no difference in concentration. The data indicate that the NAA residue is either in the skin or flesh, most likely both, and is not present on the berry surface. Due to this new information, it seems that changing wetting agent formulation is unlikely to reduce the level of residual NAA.

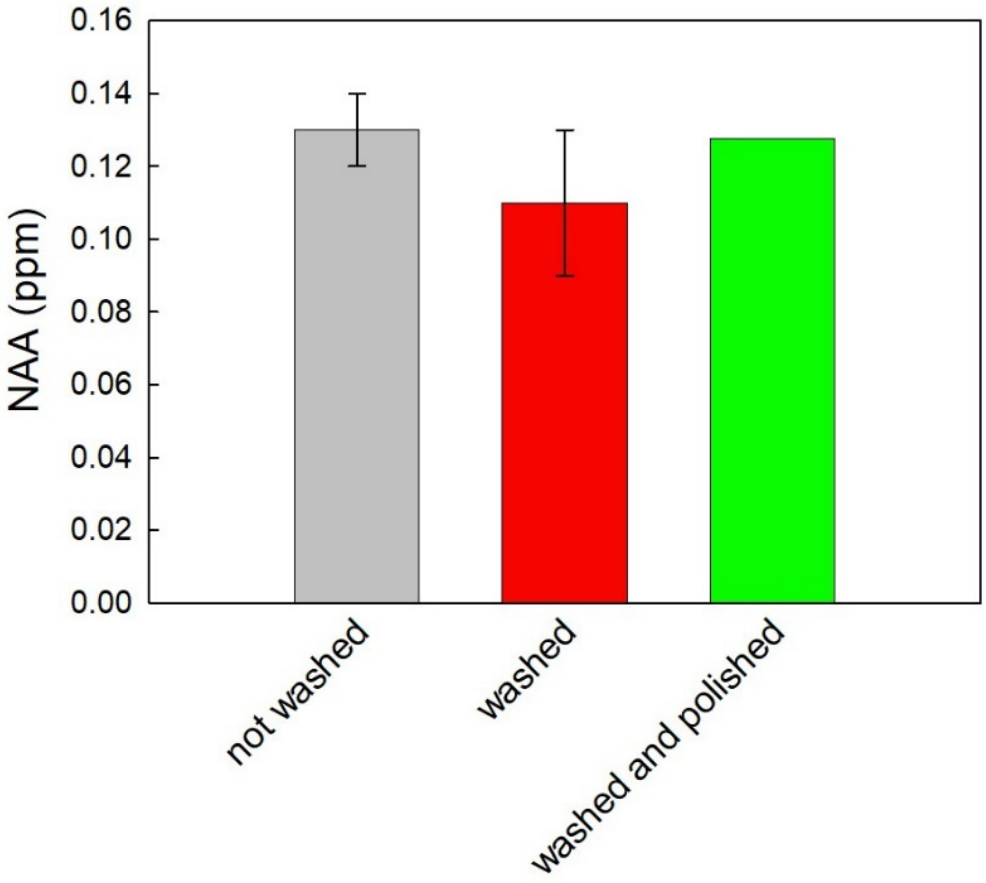


Figure 55. NAA residue in berries treated with 100 mg/L NAA ('not washed'), in berries treated with 100 mg/L NAA and then washed ('washed'), as described in Materials and Methods, and in berries treated with 100 mg/L NAA that had been washed followed by wax removal via polishing ('washed and polished'). No significant difference was detected as defined by ANOVA.

6.11 Residues in NAA-treated fruit and wines – NAA decay over time

6.11.1 Results and Discussion

During this work, NAA-treated fruit and the subsequent wines have been tested for residues. The in-house detection method that we have developed during this work is extremely sensitive. This involves the synthesis of a stable isotope-labelled NAA-aspartic acid conjugate as the internal standard for measurement by LC-MS via the labelled isotope dilution method (e.g. see Figures 8, 13, 56). This standard is produced in-house through the chemical conjugation of a stable isotope-labelled aspartic acid molecule to an NAA molecule (Böttcher *et al.,* 2011a). While this method is very senstive and not compromised by the other compounds in the matrix being tested it is not available to commercial testing facilities. This is because the standard is not commercially available. Commercial testing has to be conducted by a different method that uses unlabelled NAA as the standard molecule (external calibration). We have conducted a comparison between our in-house method and that done by a commercial company (Eurofins, Table 24). Due to the interference of the matrix in the measurements done by the commercial method, NAA was below the limit of detection in fruit samples. However, the in-house method allowed us to detect low levels of NAA in fruit samples (Figures 9, 14, 56). In wine, both methods allowed the detection of NAA residue. This is most likely because of the fermentation process reducing the amount of any compounds that were present in the fruit and that interfered in the commercial detection method. No NAA was detected in Control wine. Between 25 and 100 ppm NAA application rate the residue found in wine was roughly proprtional to the amount used for the treatments (Table 24). The two methods yielded similar results.

Table 24. NAA concentration (ppm) in experimental wines comparing concentration determined by the in-house stable-istope methods and the commercial method.* = fruit sprayed twice. CS, Cabernet Sauvignon; SH, Shiraz.

NAA conc applied (ppm)	Cultivar/year	In-house NAA (ppm)	Commercial assay NAA (ppm)
25	CS 2018	0.07	0.11
100	CS 2018	0.22	0.31
100	SH 2017*	0.81	0.53
100	CS 2017	0.51	0.60

It can be seen that the residue levels vary quite a lot between experiments (compare the two CS 100 ppm samples). The reason for this is unknown but this phenomenon is not uncommon in such studies. The fact that the NAA residues in the laboratory scale wine were low (Figure 41) suggests that extended maceration might release NAA from the skins. Further, similar information was collected from other experiments but these are the data for which we have a direct comparison between the in-house and commercial analysis methods.

To investigate the the stability of NAA residues in wine, assays were done on bottled wines over a number of years. Figure 56 shows just one example of the type of data that have been collected. Wine was made from Cabernet Sauvignon fruit treated with two concentrations of NAA (25 and 100 ppm) bottled 29/08/2018 and sampled for analysis over the next three years. Allowing for some variation in measurement, it can be seen that the NAA residue levels decrease steadily and by three years after bottling the levels are much lower. This indicates that NAA breakdown is occurring during bottled wine storage, which further decreases residue levels.

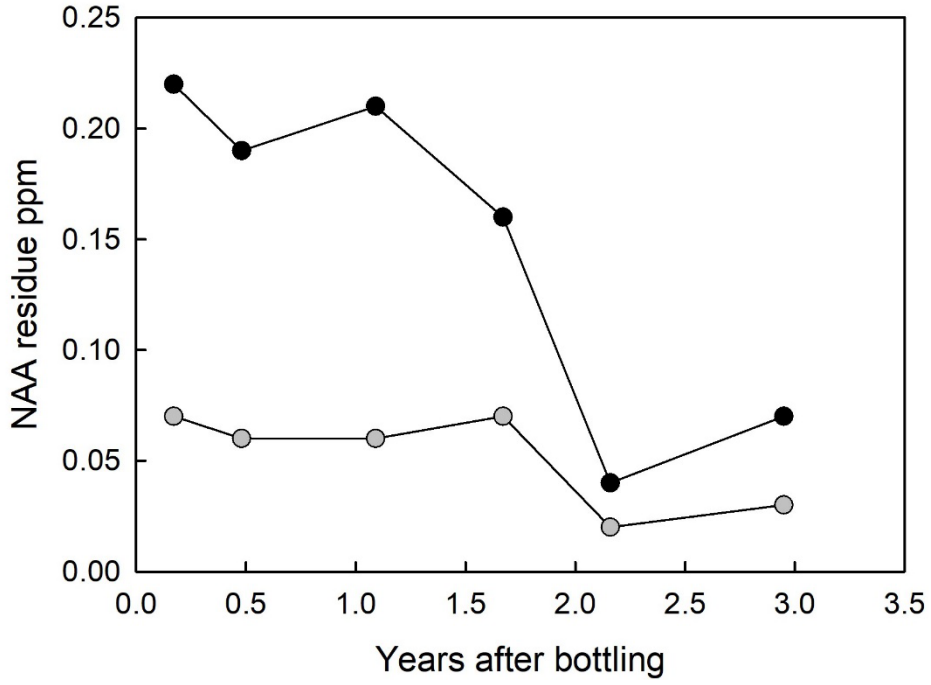


Figure 56. NAA residues in wine made from Cabernet Sauvignon berries treated with 25 ppm NAA (grey symbols) and 100 ppm NAA (black symbols).

6.12 Identification of non-volatile marker compounds for determining the most efficacious time for NAA treatment to delay berry ripening/harvest

6.12.1 Introduction

One important observation from our many studies on NAA-induced ripening delay is that the effectiveness of the NAA treatment is highly dependent on the timing of application. It seems that there exists an as yet poorly defined 'window of opportunity' during the pre-veraison phase, where NAA application needs to occur to delay ripening (see Figure 57).

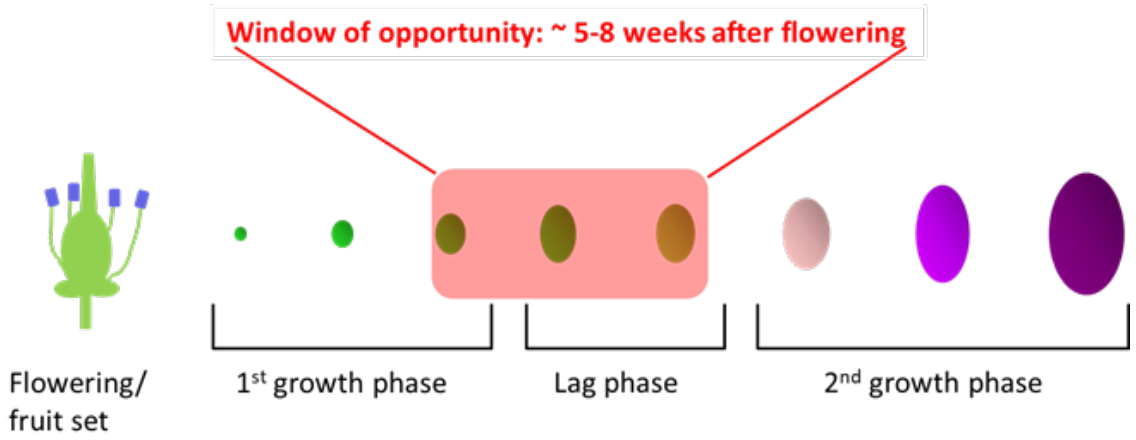


Figure 57. Schematic illustration of berry developmental stages and the 'window of opportunity' for achieving ripening delay with NAA applications.

Furthermore, our previous trials suggest that treatment timing within this window heavily influences the extent of the ripening delay that can be achieved. Some evidence shows that application closer to the time of veraison is most effective (Section 6.3), but more work is required to better define this window and ultimately to develop a quick and easy test system for growers to assist with their decision on the timing of NAA applications. Our strategy for the determination of optimal treatment timing involved the mass spectrometry-based identification of non-volatile metabolite markers in developing berries. The aim was to identify metabolites that are either present or absent (or significantly increased/decreased in their concentration) in berries at points in their development when NAA application is at its most effective, resulting in a maximum ripening delay.

This process required initial tests on Shiraz berries with NAA treatments covering the window of opportunity at close intervals to accurately assign treatment time point to ripening delay efficacy. Untreated berry samples were taken at each treatment time-point and analysed for differences in metabolite content to identify metabolites with berry accumulation patterns that can be used as markers for developmental stages corresponding to the desired efficacy of NAA treatment.

Metabolite markers identified in this way will need to be confirmed in several vintages and different climates. Different cultivars will also need to be tested as it is possible that specific, cultivar-dependent markers will be identified or that one or more markers are universal. Following on from the validation of suitable marker compounds, the next step of the process is to work towards developing a test system that can be used by growers in the field. This will involve the filtering of markers for those that can be

measured with high sensitivity in grape juice, ideally with a simple test system, e.g. by colour change of a matrix, with the long-term goal to develop a test kit.

6.12.2 Materials and methods

6.12.2.1 Trial design, sampling, and processing

Season 2015/16

Location**:** Commercial vineyard, Willunga, South Australia, Shiraz block (-35.26˚, 138.55˚).

Design and sampling: Grapes in five rows (97-101) were used for the experiment, six bunches/row (= replicate) were tagged for NAA treatments (100 mg/L in 0.1% Chemwet, 0.75 mL/L 1 N NaOH) at 4, 5, 5.5, 6, 6.5, 7, 7.5 and 8 weeks post flowering (wpf), using a different set of six bunches for each treatment time point. Veraison, defined as the last sampling time-point prior to a significant increase in total soluble solids (TSS) levels, was at 8.5 wpf. At the time of each spray, 50 untreated berries/row were sampled for snap-freezing to be used for metabolite extraction. Starting at 10 days after the 8 wpf spray, eight berries from each NAA-treated bunch were collected at regular intervals, used for the determination of individual TSS concentration and then frozen for anthocyanin measurements. At two time-points (11.5 wpf and 15 wpf) separate samples were taken for juice analysis of basic biochemical parameters.

Processing: Frozen berry samples were ground as described in the general Methods section

Season 2016/17

Location: Commercial vineyard, Willunga, South Australia, Shiraz block (-35.26˚, 138.55˚).

Design and sampling: Grapes in the same five rows as in 2015/16 were used, eight bunches/row (= replicate) were tagged for NAA treatments (100 mg/L in 0.1% Chemwet, 0.75 mL/L 1 N NaOH) at 4, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 (veraison) and 9 wpf (different set of eight bunches for each treatment time point). At the time of each spray, 70 untreated berries/row were sampled for snap-freezing to be used for metabolite extraction. For larger-scale metabolite extractions, an additional 2-3 kg of untreated berries were collected at 4, 5, 6, 6.5, 7.5 and 8 wpf and immediately frozen in liquid nitrogen. From the 8 wpf spray time-point onwards, eight berries from each NAA-treated bunch were collected at regular intervals, used to measured combined TSS and then discarded.

Processing: Frozen berry samples were ground as described in the general Methods section. Seeds were separated from skin/flesh tissue of frozen berries (4 wpf and 8 wpf) prior to grinding and metabolite extraction for tissue localisation studies and compound purification.

Season 2018/19

Location: Commercial vineyard, Willunga, South Australia, Shiraz block (-35.26˚, 138.55˚), Cabernet Sauvignon block (-35.27˚, 138.55˚).

Design and sampling: Large berry samples (2-3 kg) for metabolite extraction were collected from Shiraz and Cabernet Sauvignon at 4, 5, 7, 7.5 and 8 wpf. In addition, smaller samples of 50 berries were taken at 3-4-day intervals between 7-11 wpf to determine the point of veraison for both cultivars.

Processing: Frozen berry samples were ground as described in the general Methods section.

Season 2019/20

Location: as in season 2018/19.

Design and sampling: as in season 2018/19.

Processing: as in season 2018/19.

Determination of basic fruit quality parameters: See general Method section

6.12.2.2 Metabolomics workflow

The metabolomics workflow used in this study consisted of sample preparation, separation, data collection, feature (=compound/metabolite) finding, statistical analysis and compound identification and is further illustrated in Figure 58.

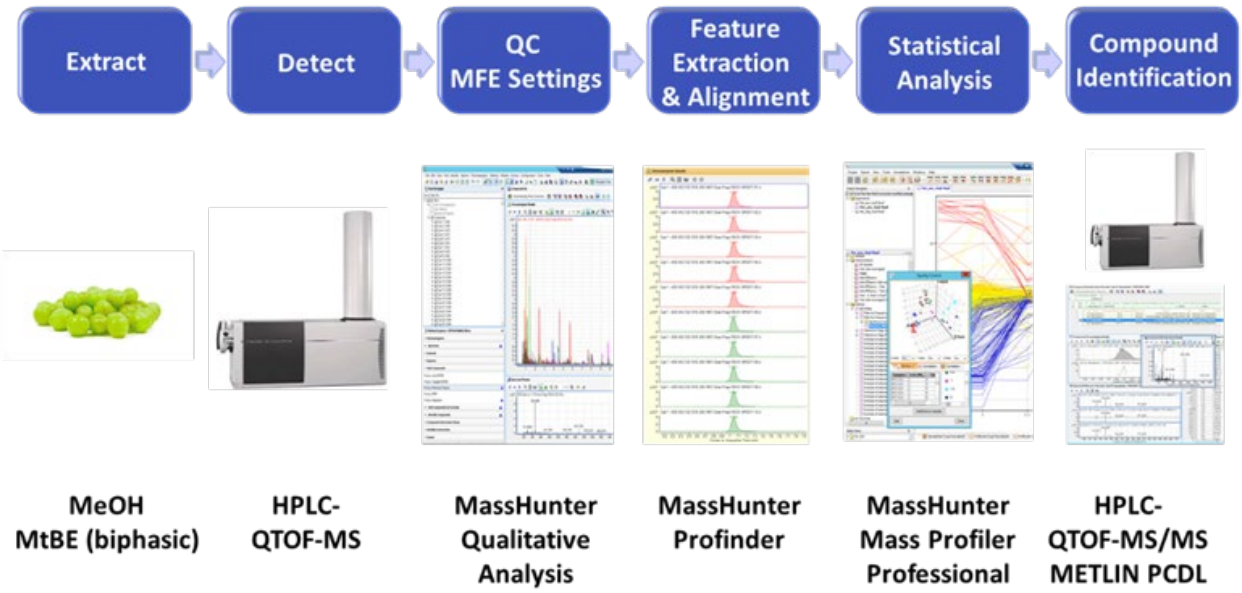


Figure 58. Metabolomics workflow as followed in this study. Grape samples were extracted with two different solvent systems, extracted metabolites were separated by HPLC and associated ions detected by QTOF-MS, three different Agilent MassHunter software packages were used to analyse the QTOF-MS data and identify significant differences between samples, and metabolite fragmentation via HPLC-QTOF-MS/MS was employed to screen databases like the METLIN PCDL with the aim to identify compounds-of-interest. HPLC, high performance liquid chromatography; MeOH, methanol; MFE, molecular feature extraction; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MtBE, methyl tert-butyl ether; PCDL, personal compound library; QC, quality control; QTOF, quadrupole time of flight.

6.12.2.3 Metabolite extraction

Methanol (MeOH) extraction

For standard extractions, 3 mL of 90% (v/v) MeOH was added to 1 g of frozen, powdered grape tissue and the sample mixed by brief vortex. Metabolite extraction was performed at 4˚C and 1250 rpm for 15 min, followed by sonication for 5 min in ice water. Samples were spun at high speed for 15 min, the supernatant was transferred to a fresh tube and dried *in vacuo* at 50˚C. The residue was dissolved in 50 µL of MeOH, followed by the addition of 50 µL of 0.4% (v/v) formic acid in water. Samples were spun at high speed for 3 min at room temperature prior to analyses via HPLC-QTOF-MS.

Methyl tert-butyl ether (MtBE) extraction

Small amounts (0.1-1 g) of frozen, powdered berry tissue were extracted with 4 times the volume of cold 80% (v/v) MeOH and 6 times the volume of MtBE at 4˚C and 1250 rpm for 30 min, followed by sonication for 10 min in ice water and addition of 6.5 times the volume of 10% (v/v) MeOH. Samples were spun at high speed at 4°C for 10 min, resulting in an upper organic phase, a lower aqueous phase, and a pellet. The two liquid phases were isolated into separate tubes and dried *in vacuo* at 55˚C.

Aqueous phase

The residue was redissolved in 100 µL deionised water, spun for 3 min at high speed and the supernatant used for analysis via HPLC-QTOF-MS.

Organic phase

The residue was dissolved in 500 µL acetonitrile (ACN)/iso-propanol (7:3 (v/v)) spun for 3 min at high speed and the supernatant used for analysis via HPLC-QTOF-MS.

6.12.2.4 Metabolite separation and detection - HPLC-QTOF-MS

The analysis of all metabolite samples from this study was performed using an Agilent 1290 Infinity series HPLC system with binary pump and degasser, autosampler with thermostat and temperature-controlled column compartment. The HPLC system was coupled to an Agilent 6530 QTOF mass spectrometer with a dual jet steam (AJS) electrospray ionisation (ESI) source operated in positive and negative ion modes. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution through a second nebuliser. The instrument method parameters are given in Table 25.

Table 25. LC and MS conditions used in this study.

LC conditions	
Instrument	1290 Infinity II
Column	Poroshell 120, SB-Aq, 2.1 x 100 mm, 2.7 µm (MeOH extract) Pororshell 120, EC-C18, 2.1 x 100 mm, 2.7 µm (Aqueous phase) Pororshell 120, EC-C8, 2.1 x 100 mm, 2.7 µm (Organic phase)
Column Temp	30°C
Injection Vol	2 µL
Autosampler Temp	6°C
Mobile Phase	A = 0.1-0.2% FA (MeOH extract; Aqueous phase) A = 1% 1 M NH_4Ac, 0.1% AA (Organic phase) B = 90% ACN/0.1-0.2% FA (MeOH extract; Aqueous phase) B = 70% ACN/30% 2-propanol (1% 1M NH_4Ac, 0.1% AA) (Organic phase)
Flow Rate	0.35-0.40 mL/min
Gradient	Variations of 5%-100%B over 13-28 min
Run Time	18-33 min
MS condition	
Instrument	Agilent 6530 Accurate Mass QTOF-MS
Ionisation Mode	Positive and negative ESI with Dual AJS
Instrument Mode	2 GHz extended dynamic range
Tune Range	Low (1700 m/z)
Autosampler Temp	6°C
Drying Gas Temp	300°C
Drying Gas Flow	8 L/min
Sheath Gas Temp	350°C
Sheath Gas Temp	11 L/min
Nebuliser Gas	40 psig
Capillary	3500 V
Fragmenter	100 V
Scan Rate	2 spectra/s

Positive and negative ions were detected in separate runs to maximise detection coverage and a minimum of two references ions were monitored throughout each run to ensure mass accuracy.
All samples from one season were run and analysed as a batch and each batch included double blank samples, process blank samples and ten quality control (QC) samples. The QC samples represented an equal-proportion mixture of all berry samples included in a batch and were used to determine appropriate analysis parameters for downstream sample processing and one QC sample was injected after every five runs of a batch to monitor instrument performance.

6.12.2.5 Data analysis

Feature finding – MassHunter Qualitative Analysis
Compounds were extracted from the raw data files using an unbiased Molecular Feature Extraction (MFE) algorithm in Agilent MassHunter Qualitative Analysis software (B.07.00). The MFE algorithm selects all ions that are related, including isotopes and adducts and then sums all ion signals into one value, termed 'molecular feature'. The QC sample runs were used to assess the quality of the data and to determine suitable settings (e.g. noise level, peak height) that balanced the number of extracted features (1000-4000 across all the samples run in this study) with the highest possible quality of the mass spectrometry profiles of those features (e.g. ion number, isotope patterns). The optimised settings were then applied to all batch sample data files for feature extraction via recursive batch analysis in MassHunter Profinder (B.06.00).

Feature extraction – Recursive batch analysis in Profinder
This program combines MFE with the Find by Ion (FbI) algorithm: MFE extracts features and performs binning and Rt alignment across all batch files and FbI re-extracts, integrates and scores all features, reducing false positives and false negatives. The settings for MFE extraction parameters used were predetermined by QC sample analysis. The batch analysis output was manually curated and exported for use.

Statistical analysis and compound identification– Mass Profiler Professional
The processed data files were subsequently analysed using Agilent Mass Profiler Professional (MPP, B13.11), a multi-dimensional statistical analysis and data visualisation software. Principal component analysis (PCA) and hierarchical clustering were used to assess the quality of the replicated data profiles and to observe general metabolite abundance pattern changes over time. Provisional compound identification was performed by matching accurate mass results to content from the METLIN Personal Compound Database and Library (PCDL). In addition, MS/MS spectra for selected compounds of interest were acquired (for MS/MS conditions, see Table 26) and queried against the METLIN spectral database as well as the Global Natural Products Social Molecular Networking (GNPS) database (https://gnps.ucsd.edu/), a web-based mass spectrometry ecosystem that aims to be an open-access knowledge base for community-wide organisation and sharing of raw, processed, or annotated fragmentation mass spectrometry data. In cases where MS/MS data of sufficient quality were collected, but could not be matched, Agilent Molecular Structure Correlator (MSC) software was used to further interrogate the spectra with the aim to putatively identify compounds.

Table 26. MS/MS conditions used in this study.

MS/MS conditions	
Acquisition Mode	Auto MS/MS
Scan Rate MS	2 spectra/s
Scan Rate MS/MS	2-4 spectra/s
Isolation Width	Medium (~4 m/z)
Collision Energy	10, 20, 40 V
Maximum Precursors/Cycle	1-3

The process of identifying compounds-of-interest involved two different approaches of statistical analysis of the metabolite data: 1) Analysis of Variance (ANOVA) to identify compounds with a minimum significant ($P < 0.05$) abundance difference of 10-fold between sampling time points or 2) Pearson correlation ($P < 0.05$) to screen for compounds with accumulation patterns that showed a high degree of correlation (either negative or positive) with the degree of ripening delay efficacy determined for the NAA treatments.

6.12.2.6 Metabolite purification

Extraction

Metabolite extractions were performed as described in 6.12.2.3 but scaled up for 30 g – 1 kg of grape material.

HPLC separation – Round 1

The HPLC parameters shown in Table 27 represent an example of round 1 conditions that were used for metabolite separation. Many variations on these conditions were employed to optimise the separation of compounds-of-interest.

Table 27. Example of round 1 HPLC conditions used in this study.

HPLC conditions	
Instrument	Hewlett Packard 1100 series with quarternary pump, autosampler DAD detector and automatic fraction collector
Column	Phenomenex Luna 5 µm C18(2) 100 Å; 250 x 4.6 mm
Column Temp	30°C
Injection Vol	25 µL
Autosampler Temp	RT
Mobile Phase	A = 0.2% FA in water B = 0.2% FA in ACN
Flow Rate	1 mL/min
Gradient	5%-20%B in 10 min, 20%-30%B in 5 min, 30%-100%B in 10 min, hold for 30 min, then back to 5%B in 1 min
Run Time	35 min

HPLC separation – Round 2

The HPLC parameters provided in Table 28 are representing an example of round 2 conditions that were used for metabolite separation. Many variations of these conditions were employed to optimise the separation of compounds-of-interest.

Table 28. Example of round 2 HPLC conditions used in this study.

HPLC conditions	
Instrument	Hewlett Packard 1100 series with quarternary pump, autosampler DAD detector and automatic fraction collector
Column	Phenomenex Luna 5 µm C18(2) 100 Å; 250 x 4.6 mm
Column Temp	30˚C
Injection Vol	25 µL
Autosampler Temp	RT
Mobile Phase	A = 3% (v/v) ACN, 0.2% FA in water B = 3% (v/v) ACN, 0.2% FA in water
Flow Rate	1 mL/min
Gradient	isocratic
Run Time	40 min

6.12.2.7 Large-scale glass column chromatography

The glass column used for separating extracts of up to 1 kg of grape material was custom made (Glassworks, Victoria University of Wellington, NZ) and contained a 500 mL reservoir and 300 mL bed volume. The column was loaded with 200 g of stationary matrix, e.g. Diaion HP-20 resin, using cotton wool as a frit.

Below is an example of the extraction, loading and elution procedure of which many variations were used in this study:
500 g of grape tissue was extracted with 1 L of MeOH by a 5 min sonication in ice water, followed by 15 min of agitation at 4˚C. The extract was spun down and the supernatant transferred to a pre-cooled glass vessel. The residue was re-extracted with 500 mL of 70% (v/v) MeOH and combined with the initial supernatant. The column resin was pre-conditioned with 600 mL of MeOH, followed by 600 mL of 70% (v/v) MeOH, before loading the grape extract. The eluent was collected and slowly diluted with an equal volume of water after which it was passed through the column again. This process was repeated two more times, then the column was washed with 600 mL of water. The elution was performed with 600 mL each of increasing concentrations of ACN (5%, 10%, 20%, 30%, 50% and 100%) and each fraction was tested for the presence of compounds-of-interest using HPLC-QTOF-MS as described above.

6.12.2.8 Nuclear magnetic resonance (NMR) analysis

All NMR analyses were performed by Dr. Rob Keyzers, Victoria University of Wellington.

6.12.3 Results and discussion

6.12.3.1 Initial screen for potential NAA efficacy metabolite markers – Shiraz field trials

Prior to the project, internally funded Shiraz trials were performed to establish the optimal time-points for NAA treatment when aiming for small to maximum ripening delays and to provide material for the subsequent development of methods for the extraction and identification of compounds associated with the respective treatment time points.

High frequency pre-veraison NAA sprays link treatment time to ripening delay efficacy

Five sets of six (2015/16) and five sets of eight (2016/17) Shiraz bunches were treated with 100 ppm NAA at eight (2015/16) or ten (2016/17) time-points, spanning late stages of growth phase 1, the lag phase and very early stages of growth phase 2, to accurately determine the optimal treatment time for different degrees of ripening delay. Sugar accumulation of treated grapes was monitored from veraison through to commercial maturity to quantify the resulting delay in ripening. In both seasons, veraison, defined as the last sampling time point before a significant increase in sugar accumulation was observed, was determined to be at 8.5 weeks post flowering (wpf). Mid-ripening total soluble solids (TSS) measurements were used to rank treatment time-points according to their ripening delay efficacy (Tables 29 and 30). In 2015/16, basic biochemical grape juice parameters measured with an OenoFoss instrument at 11.5 wpf were also considered in the assessment of berry developmental stages (Table 29). In both seasons, a stepwise increase in ripening delay was observed from the early lag-phase (5.5-6.5 wpf) treatments to the last treatment time point prior to veraison (8 wpf).

Table 29. Basic grape parameters of NAA-treated fruit during the 2015/16 season. Two ripening indicators at 11.5 wpf (pH, TA) and TSS at 13 wpf were used to deduce ripening delay efficacy scores. Values are the mean of $n = 5$ biological replicates and different letters indicate significant difference by one-way ANOVA ($P < 0.05$); N/A, not applicable.

Treatment time-point (wpf)	OenoFoss data			Ripening delay efficacy [scale of 1 (low) to 4 (high)]
	pH	TA 8.2 (g/L)	TSS (˚Brix)	
4	3.40a	8.68d	17.94ab	1
5	3.36ab	9.40d	17.30abc	1
5.5	3.32abc	10.20cd	17.54ab	2
6	3.34abc	10.80bcd	17.19abc	2
6.5	3.29abc	11.34bcd	16.54bc	3
7	3.27bc	12.74bc	16.60bc	3
7.5	3.26bc	13.28b	16.46bc	3
8	3.25c	16.08a	15.12c	4
untreated	-	-	19.36a	N/A

Table 30. TSS of NAA-treated and untreated berries during the 2016/17 season. Measurements were taken at 12.5 wpf and were used to deduce ripening delay efficacy scores. Values are the mean of *n* = 5 biological replicates and different letters indicate significant difference by one-way ANOVA (*P* < 0.05); N/A, not applicable.

Treatment time-point (wpf)	TSS (˚Brix)	Ripening delay efficacy [scale of 1 (low) to 3 (high)]
4	17.2ab	1
5	16.8ab	1
5.5	15.9b	2
6	15.5bc	2
6.5	15.3bc	2
7	14.9bc	2
7.5	14.7bc	2
8	13.1c	3
8.5 (veraison)	16.4ab	1
9	16.6ab	1
untreated	18.7a	N/A

Earlier treatment time-points (4 and 5 wpf) did not result in a significant ripening delay when compared with untreated fruit in both seasons and the veraison and post-veraison treatment time-points added in 2016/17 also failed to induce a significant delay in berry sugar accumulation. Anthocyanin measurements taken in 2015/16 generally agreed with the sugar- and acidity-based ripening efficacy rankings, however, large variation between biological replicates meant that only the 8 wpf treatment time-point was confirmed to result in a significant delay in anthocyanin accumulation (data not shown). This variability, as well as the clear ripening delay in fruit treated at 8 wpf, is also apparent in photos of representative bunches, taken at 10.5 wpf (Figure 59).



Figure 59. Photos of representative Shiraz bunches treated with NAA at eight different time-points in 2015/16. All photos were taken on Jan 12, 2016, 10.5 wpf.

6.12.3.2 Berry metabolite extracts and compound-of-interest discovery workflow

Compound extraction, separation, and detection

At the time-points of NAA application, samples of untreated grapes located in the same rows as the treated bunches, were collected, frozen, processed to a powder, and used for metabolite extractions. For the 2015/16 season this amounted to 40 berry samples (eight time-points, five replicates), which increased to 50 berry samples in the 2016/17 season (ten time-points, five replicates). In order to maximise the recovery of metabolites with highly diverse properties, berry stage samples, together with QC samples comprising a mixture of all collected sample in one season, were subjected to two different types of extraction: 1) extraction with polar solvents (methanol (MeOH)/water) or 2) extraction with a mixture of non-polar (methyl tert-butyl ether, MtBE) and polar (MeOH/water) solvents, resulting in phase separation (aqueous and organic) and further analyses of both phases. Extracts were subjected to different reverse phase HPLC separations and subsequent QTOF-MS analyses in positive and negative ion modes; in both seasons and 1000-4000 high quality molecular features, each representing a unique compound, were identified for each extraction and ionisation mode. An extracted compound chromatogram (ECC) example of the aqueous phase of a 2016/17 QC MtBE extract is shown in Figure 60.

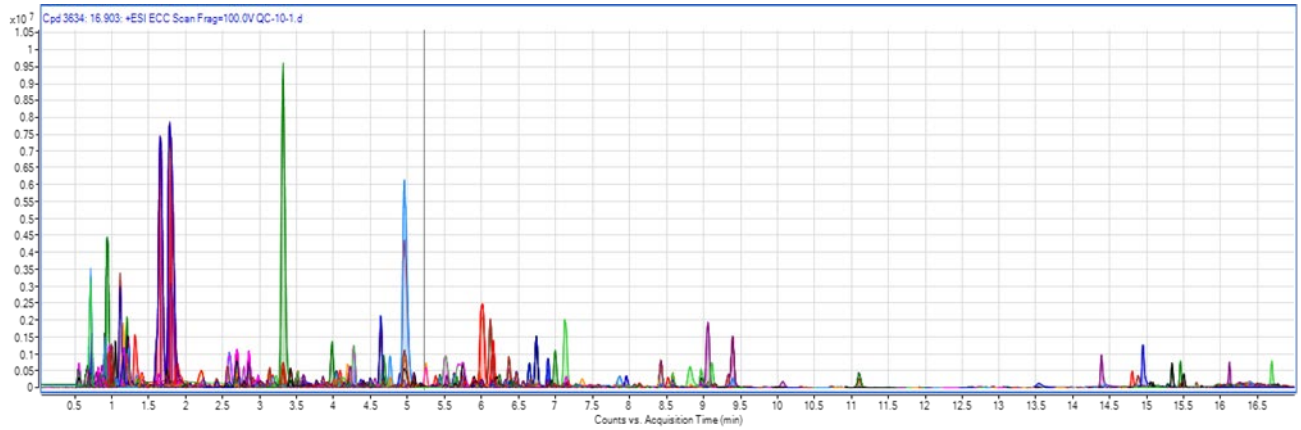


Figure 60. Extracted ion chromatogram (ECC) of a 2016/17 QC sample. The aqueous phase of a MtBE extraction was separated on a C18 column using a gradient of ACN and water acidified with formic acid. Ions were detected by QTOF-MS in positive ionisation mode. Agilent MassHunter Qualitative Analysis software was used to extract 3634 molecular features (= metabolites), each represented by a chromatogram peak.

Screening for compounds-of-interest

Acquired and curated data from MeOH extracts and the aqueous and organic phases from MtBE extracts from both seasons were used to screen for compounds-of-interest via the statistical analysis and data visualisation software Mass Profiler Professional (MPP). All data were filtered to only include metabolites that were present in at least four out of the five biological replicates. Principal Component Analysis (PCA) of all extraction and ionisation modes showed that samples grouped by sampling time-point/berry stage and confirmed good reproducibility for the biological replicates in both seasons, as shown for representative MeOH extracts run in positive ionisation mode in Figure 61. The PCAs of the log2-transformed metabolite abundances explained 60.5% (2015/16) and 67.7% (2016/17) of the data variation with principal component 1 (X-Axes) as the main contributor (44% 2015/16, 53% 2016/17).

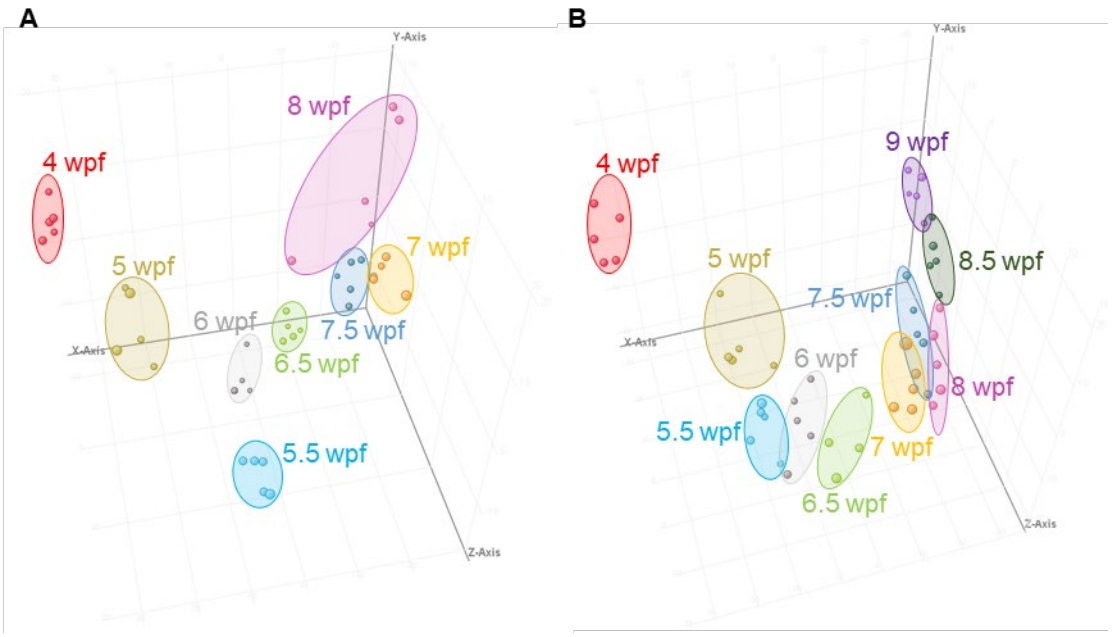


Figure 61. PCA plots of (A) 2015/16 and (B) 2016/17 MeOH extract metabolite samples. Each circle represents a replicate, and each colour represents a sampling time point.

Hierarchical clustering based on sampling time-points and extracted metabolites (referred to as 'features' in MPP) with significant difference between sample time-points (ANOVA, $P < 0.05$) further revealed that changes in metabolite/feature abundance between time-points were largely gradual. However, as illustrated by the cluster examples shown in Figure 62, a major change in the abundance of a large proportion of berry metabolites occurred between 6 wpf and 6.5 wpf in 2015/16 (Figure 62A) and between 6.5 wpf and 7 wpf in 2016/17 (Figure 62B), clearly marking an as yet uncharacterised developmental transition during the lag phase. This transition was reflected in all extracts and ionisation modes and therefore seemed to be a universal phenomenon across metabolites of different structures, polarities, and functions.

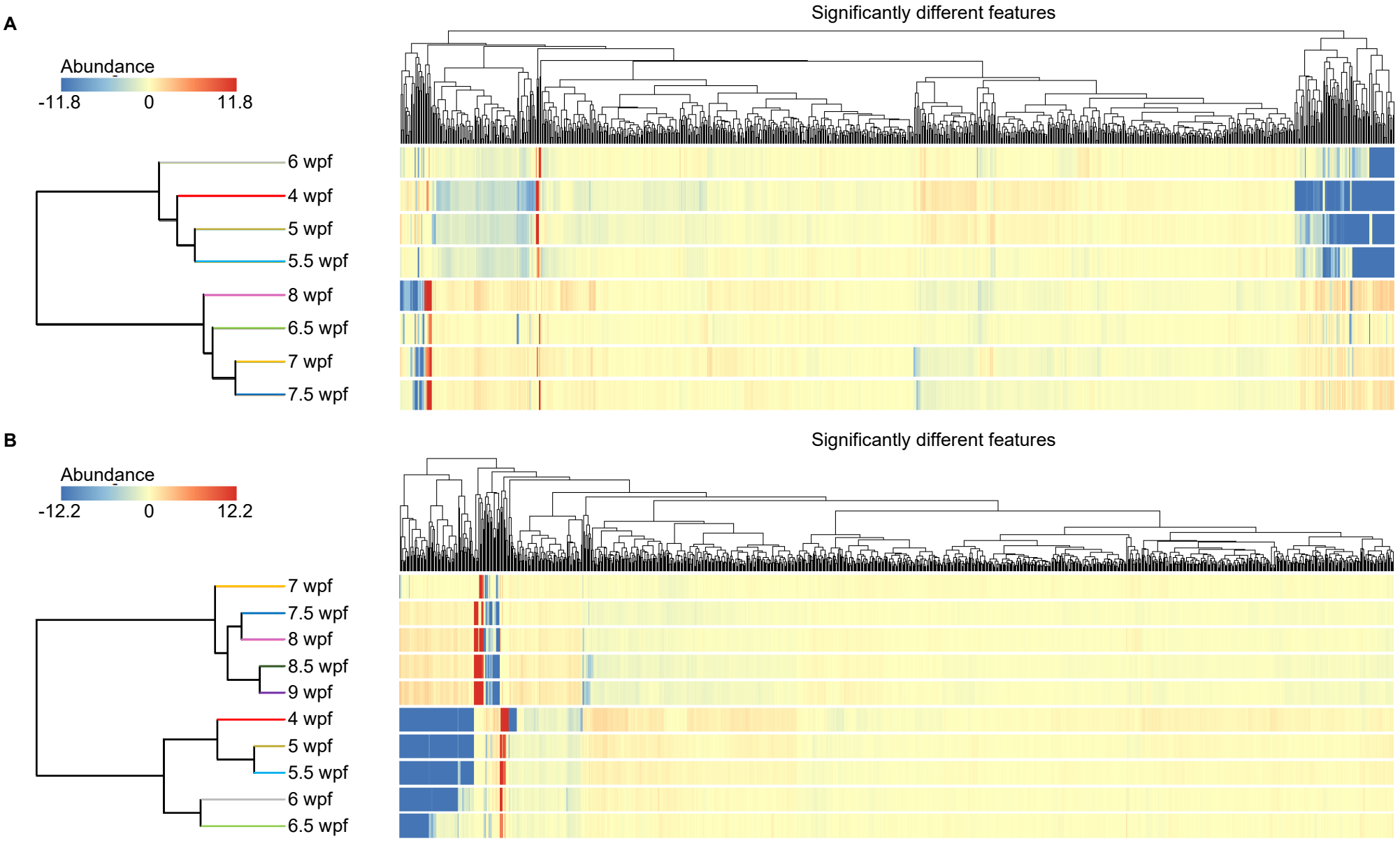

A
Abundance
-11.8
0
11.8
6 wpf
4 wpf
5 wpf
5.5 wpf
8 wpf
6.5 wpf
7 wpf
7.5 wpf
Significantly different features
B
Abundance
-12.2
0
12.2
7 wpf
7.5 wpf
8 wpf
8.5 wpf
9 wpf
4 wpf
5 wpf
5.5 wpf
6 wpf
6.5 wpf
Significantly different features

Figure 62. Hierarchical cluster analysis based on abundance profiles of metabolites/features in (A) 2015/16 and (B) 2016/17. The shown examples represent MtBE extracts (organic phase) that were analysed with HPLC-QTOF-MS in negative ionisation mode.

In order to identify potential marker compounds for NAA treatments, two different statistical methods were applied: 1) ANOVA ($P < 0.05$), identifying compounds that were significantly different and had a changed abundance of at least 10-fold, in berry stages of high or low responsiveness to NAA treatments (see Figure 63), 2) Pearson correlation using the NAA efficacy ranking (see Tables 29 and 30) and compound abundance levels to identify accumulation patterns that showed significant correlation (either negative or positive) with the degree of ripening delay efficacy determined for the corresponding NAA treatments (see Figure 63).

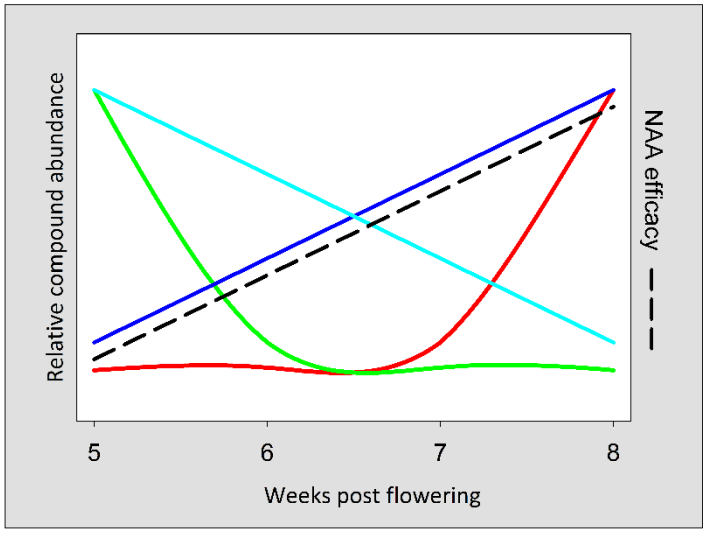


Figure 63. Idealised berry metabolite abundance profiles for NAA efficacy prediction. Red and green lines indicate metabolites with significantly increased abundances at berry stages of high or low responsiveness to NAA treatments. Light and dark blue lines represent compounds with abundance patterns that correlate, either positively or negatively, with the degree of ripening delay efficacy determined for the corresponding NAA treatments and represented by the black, dashed line.

This resulted in the identification of 163 (2015/16) and 137 (2016/17) potential marker compounds, 48 of which were shared between the two seasons (Table 31).

Table 31. Numbers and attributes of potential marker compounds for high efficacy NAA application timing in Shiraz identified in two growing seasons.

Compound attributes	Compound number (2015/16)	Compound number (2016/17)
Potential marker compound	163	137
MeOH extract positive ion polarity	48	9
MeOH extract negative ion polarity	22	12
MtBE extract, aqu. phase, positive ion polarity	23	28
MtBE extract, aqu. phase, negative ion polarity	2	32
MtBE extract, org. phase, positive ion polarity	40	20
MtBE extract, org. phase, negative ion polarity	29	38
Common in both seasons	48 (29%)	48 (35%)
High abundance at most effective spray time	93	83
Low abundance at most effective spray time	48	48
Abundance not related to spray time efficiency	22	6

Strategies to identify compounds-of-interest

The biggest challenge in any untargeted metabolomics study is to identity the features that are filtered out as being of further interest. We found that accurate mass data, as generated by the QTOF instrument, was not sufficient to unequivocally identify metabolites as database searches resulted in either no, or multiple, possible matches. The only certain information about the 300 potential marker compounds was that their mass, ionisation and isotope distribution patterns aligned with signatures of small organic compounds. We therefore sought to generate MS/MS spectra for all marker candidates to be able to access another level of compound identification analysis. Compound identification using MS/MS spectral matching provides the highest confidence possible when using mass spectrometry to identify compounds. The Agilent METLIN metabolite MS/MS library contains high quality Agilent QTOF spectra for about 2000 metabolites and provides the best chance of compound identification. MS/MS spectra of the 300 features of interest were acquired and queried in terms of accurate mass and MS/MS spectra against the METLIN metabolite PCDL, but only one of them could be matched and was identified as epicatechin (Figure 64). We then ran and MS/MS-fragmented nearly 400 organic compounds on our QTOF system that were available from the High Value Group chemical collection and were largely lacking MS/MS spectra in the METLIN library and created an in-house library containing the accurate mass data and fragmentation spectra of these compounds. However, screening the potential marker compounds against this in-house library did not return any further matches. The same result was achieved when submitting all MS/MS data to the Global Natural Products Social Molecular Networking (GNPS) database (https://gnps.ucsd.edu/), which contains over 1500 public MS/MS datasets comprising more than 80,000 spectra. As for the METLIN and in-house libraries, epicatechin emerged as the only positive identification.

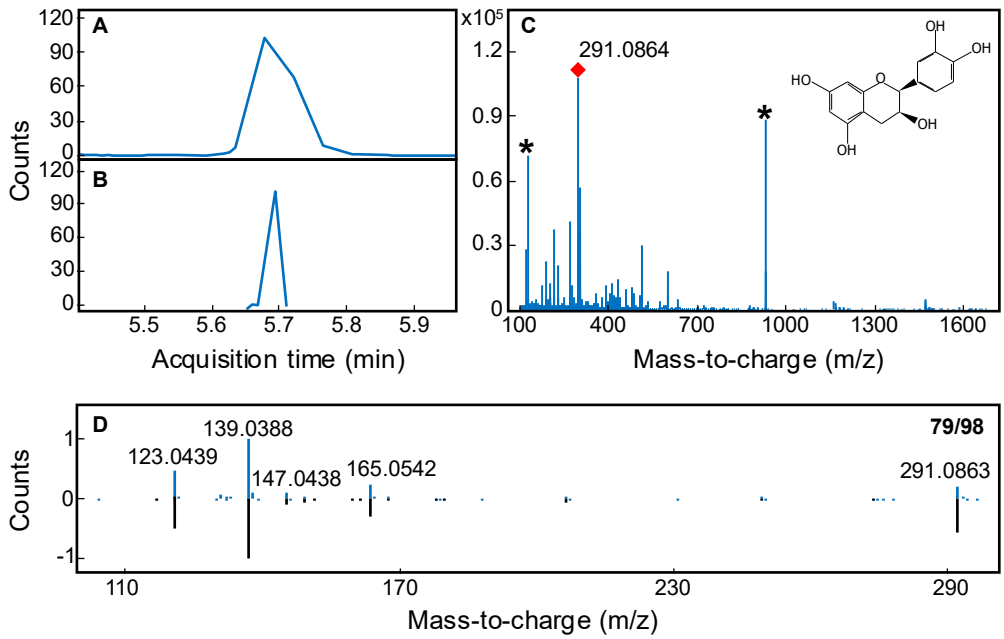


Figure 64. MS/MS-based identification of the potential marker compound epicatechin. The (A) extracted ion chromatogram (EIC), (B) ECC and (C) MS spectrum of a compound-of -interest ([M+H] ion labelled with red diamond, reference ions with black asterisks) were detected in 2015/16 Shiraz grape MeOH extracts and matched to epicatechin via a (D) METLIN PCDL search of the corresponding MS/MS spectrum acquired at a collision energy of 10 V. Grape extract fragment ions are shown in blue, METLIN PCDL ions are shown in black; forward (matching of acquired ions to library spectrum) and reverse (matching of library ions to acquired spectrum) scores are shown in bold black font. The epicatechin structure is displayed in (C).

Further attempts to analyse the collected MS/MS spectra with the Molecular Structure Correlator software, which correlates accurate mass MS/MS fragment ions for a compound of interest with one or more proposed molecular structures for that compound, were unsuccessful (data not shown). There are two possible reasons for the failure to identify 299 out of 300 compounds-of-interest: 1) either accurate mass information or MS/MS spectra, or both, are missing from the libraries used for screening for the compounds in question, or 2) the quality of the acquired MS/MS spectra is not sufficient to enable reliable matching. The latter could be caused by the low abundance of many of the potential marker compounds in the berry extracts.

All further investigations regarding the suitability of grape metabolites to serve as spray timing markers and the determination of the chemical structure of potential marker compounds to enable the development of in-field marker detection systems, were focussed on the 48 compounds that were identified as potential markers in both seasons. This excluded epicatechin as it did not meet the compound-of-interest selection criteria in the 2016/17 sample batch, which left us with 48 unidentified compounds (Table 32).

Table 32. Extraction and mass details of 48 potential marker compounds that were identified in both seasons.

Compound#	Extract	Detected mass		MS/MS spectra (Y/N)	Library match (Y/N)
		2015/16	2016/17		
1	MeOH/Aqu. phase	111.069	111.0701	Y	N
2	MeOH/Aqu. phase	145.0538	145.0532	Y	N
3	Organic phase	210.0122	210.0128	Y	N
4	Organic phase	239.0009	239.0016	Y	N
5	MeOH/Aqu. phase	264.1363	264.1349	Y	N
6	Organic phase	270.0962	270.0978	Y	N
7	MeOH/Aqu. phase	282.1475	282.1467	Y	N
8	Organic phase	282.9902	282.9916	Y	N
9	Organic phase	287.0104	287.0113	Y	N
10	MeOH/Aqu. phase	294.0957	294.096	Y	N
11	MeOH/Aqu. phase	320.0547	320.0536	Y	N
12	MeOH/Aqu. phase	372.0464	372.0479	Y	N
13	Organic phase	374.0173	374.0184	Y	N
14	MeOH/Aqu. phase	377.1837	377.1831	Y	N
15	MeOH/Aqu. phase	414.1723	414.173	Y	N
16	MeOH/Aqu. phase	424.1936	424.1936	Y	N
17	MeOH/Aqu. phase	436.1011	436.1005	Y	N
18	Organic phase	456.0206	456.0214	Y	N
19	Organic phase	470.0362	470.037	Y	N
20	MeOH/Aqu. phase	498.1556	498.1558	Y	N
21	Organic phase	552.0391	552.0402	Y	N
22	Organic phase	873.7796	895.7631	Y	N
23	Organic phase	909.7767	909.7763	Y	N
24	Organic phase	910.6486	910.6481	Y	N
25	Organic phase	911.7596	911.7563	Y	N
26	Organic phase	911.7935	911.7917	Y	N
27	Organic phase	913.7686	913.7688	Y	N
28	Organic phase	916.7128	916.7119	Y	N
29	Organic phase	927.7502	927.7512	Y	N
30	Organic phase	946.7449	946.747	Y	N
31	Organic phase	952.7819	952.7834	Y	N
32	Organic phase	989.7384	989.7374	Y	N
33	Organic phase	721.564	721.5614	Y	N
34	MeOH/Aqu. phase	384.165	384.1609	Y	N
35	MeOH/Aqu. phase	404.1683	404.1691	Y	N
36	MeOH/Aqu. phase	416.1682	416.1679	Y	N
37	MeOH/Aqu. phase	454.0753	454.0745	Y	N
38	MeOH/Aqu. phase	196.0744	196.0758	Y	N
39	MeOH/Aqu. phase	299.1737	299.1786	Y	N
40	Organic phase	117.9801	117.9809	Y	N
41	Organic phase	292.0142	292.0146	Y	N
42	MeOH/Aqu. phase	349.1031	349.1009	Y	N
43	MeOH/Aqu. phase	383.0487	383.0486	Y	N
44	Organic phase	388.0333	388.0339	Y	N
45	MeOH/Aqu. phase	397.1626	397.1613	Y	N
46	MeOH/Aqu. phase	402.1158	402.1151	Y	N
47	Organic phase	448.3575	448.3577	Y	N
48	MeOH/Aqu. phase	478.1749	478.1771	Y	N

Filtering of identified NAA efficacy markers based on suitability for juice-based detection

As outlined in the introduction, the long-term vision of this study was to identify metabolite markers that are suitable for the development of a simple detection system that could be used by growers to assess the spray-readiness state of their grapes for desired NAA-induced ripening delay.

It was therefore decided to exclude those potential marker compounds that would require extraction from the grape juice into a non-polar solvent, i.e. all compounds that were detected in the organic phase of the MtBE extracts (see Table 32), from any further investigations. This included two compounds with high abundance in grapes at low efficacy spray time-points and low abundance at high efficacy spray time-points (Figure 65) and 23 compounds that had the reverse abundance profile (two examples shown in Figure 66).

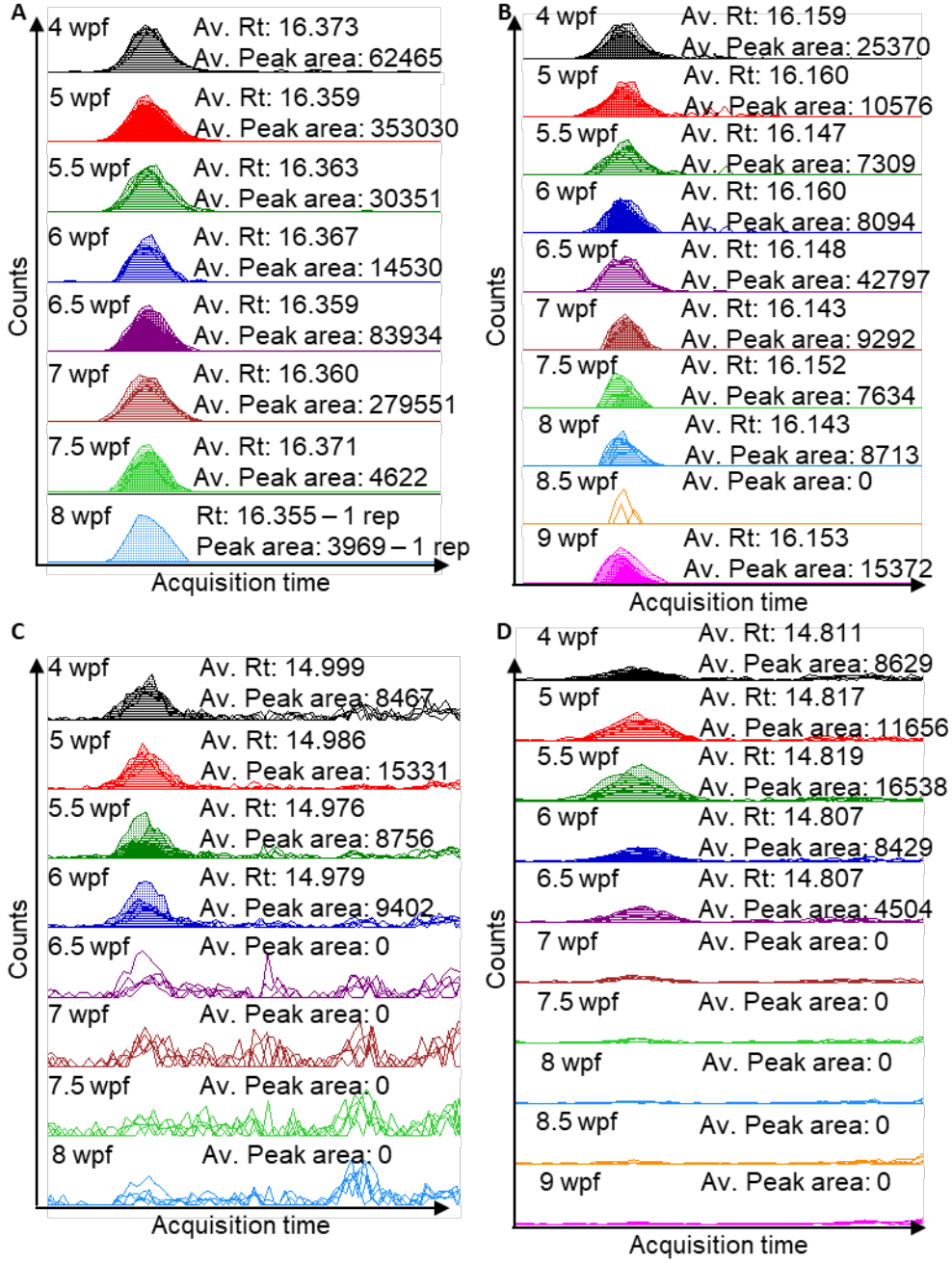


Figure 65. Extracted compound chromatograms (ECCs) of MtBE-extracted metabolites with high abundance in Shiraz grapes at low efficacy spray time-points. (A) and (C) show the overlaid, integrated ECC data for two example compounds from 2015/16 extracts. (B) and (D) display ECCs for the same two compounds from 2016/17 extracts.

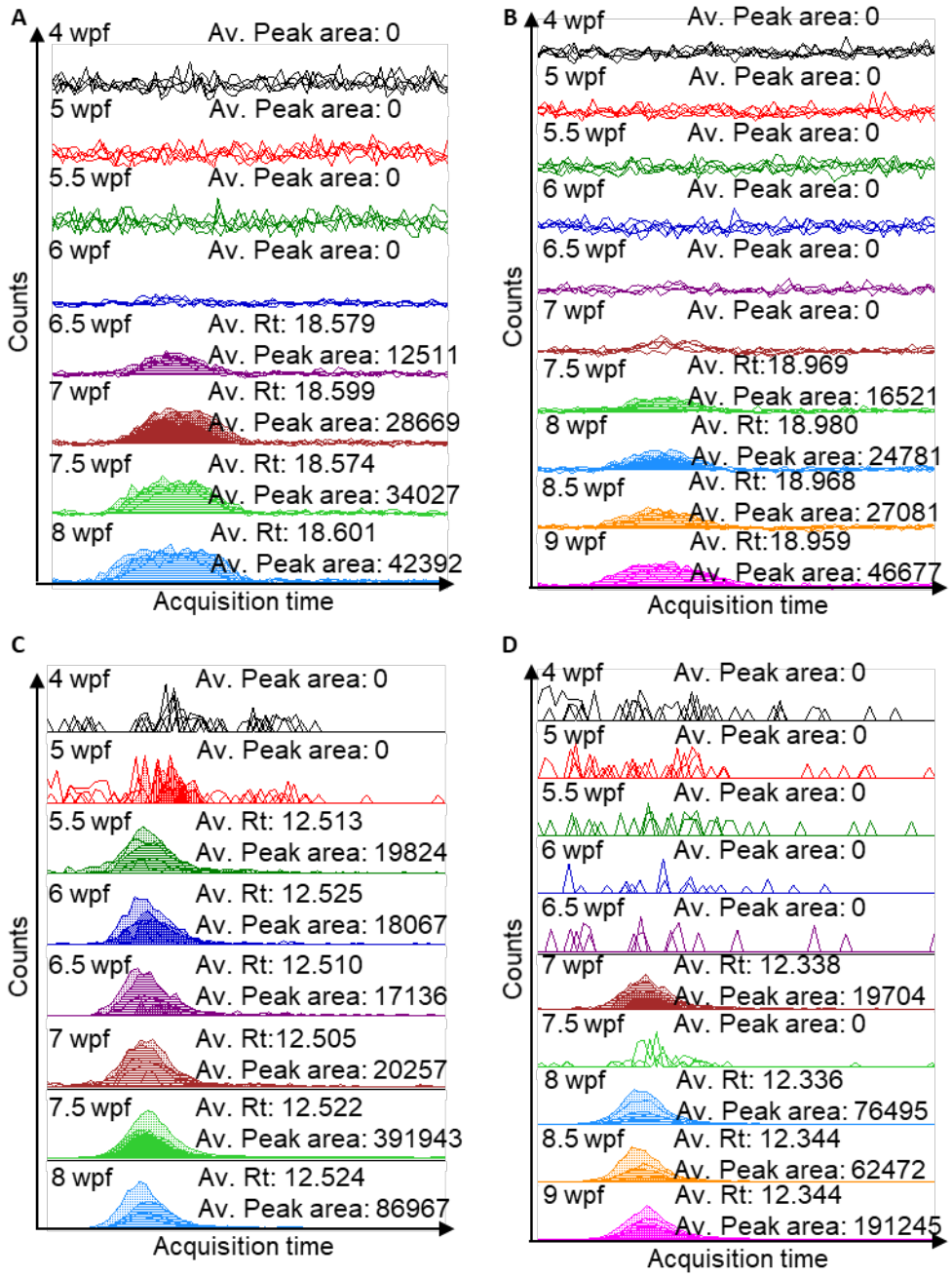


Figure 66. Extracted compound chromatograms (ECCs) of MtBE-extracted metabolites with high abundance in Shiraz grapes at high efficacy spray time-points. (A) and (C) show the overlaid, integrated ECC data for two example compounds from 2015/16 extracts. (B) and (D) display ECCs for the same two compounds from 2016/17 extracts.

The remaining 23 compounds were characterised by their solubility in water, as they were detected in the aqueous phase of the MtBE extracts and in samples extracted with MeOH (see Table 33). The relatively high polarity of these putative metabolites therefore makes them good candidates for detection

in grape juice. Out of this narrowed group of 23 compounds-of-interest, 14 compounds displayed abundance patterns that correlated negatively with the high efficacy spray time-points (two examples shown in Figure 67), whereas the abundance profiles of nine compounds were positively correlated with the high efficacy spray time-points (two examples shown in Figure 68).

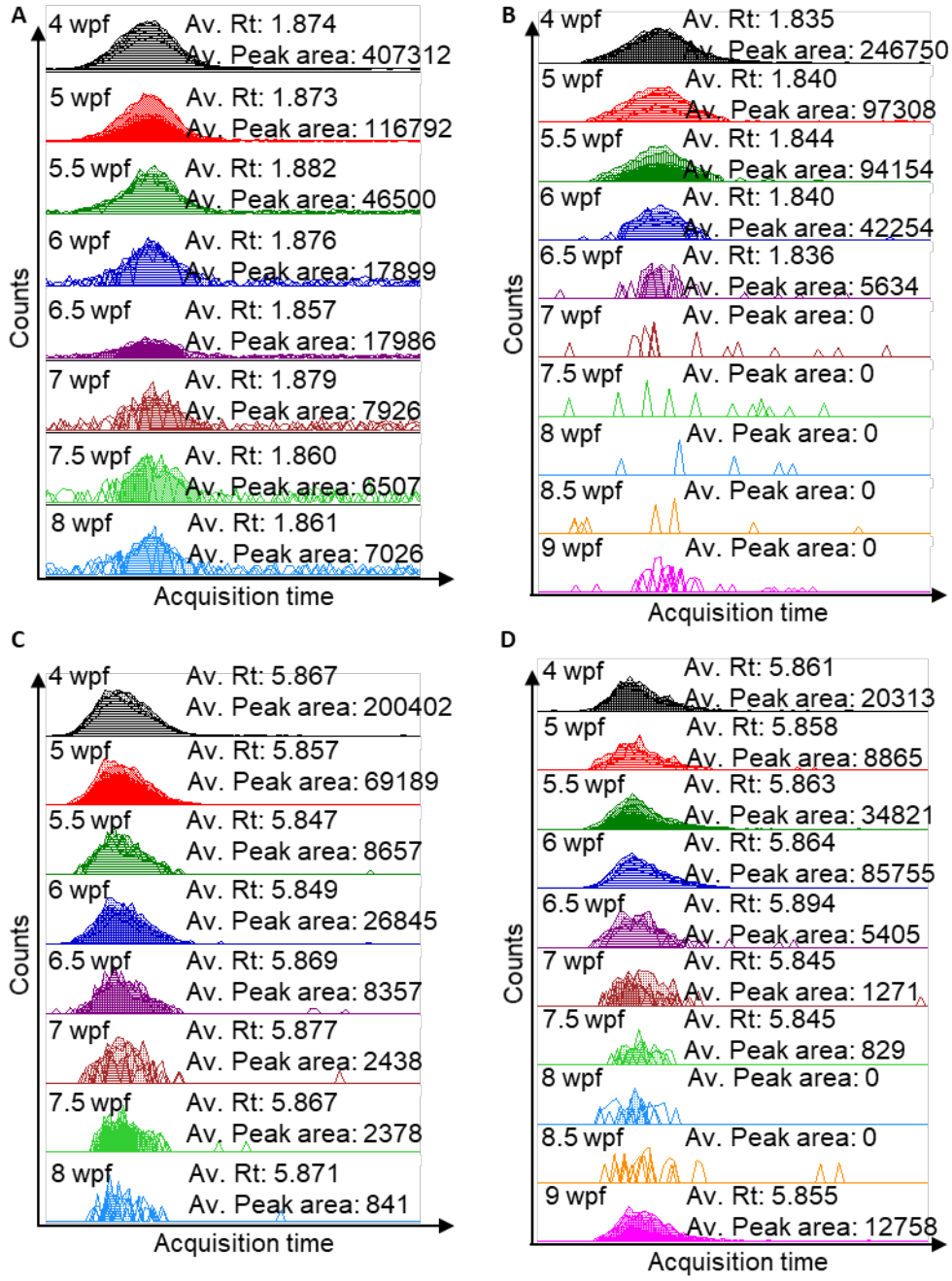


Figure 67. Extracted compound chromatograms (ECCs) of MeOH/water-extracted metabolites with high abundance in Shiraz grapes at low efficacy spray time-points. (A) and (C) show the overlaid, integrated ECC data for two example compounds from 2015/16 extracts. (B) and (D) display ECCs for the same two compounds from 2016/17 extracts.

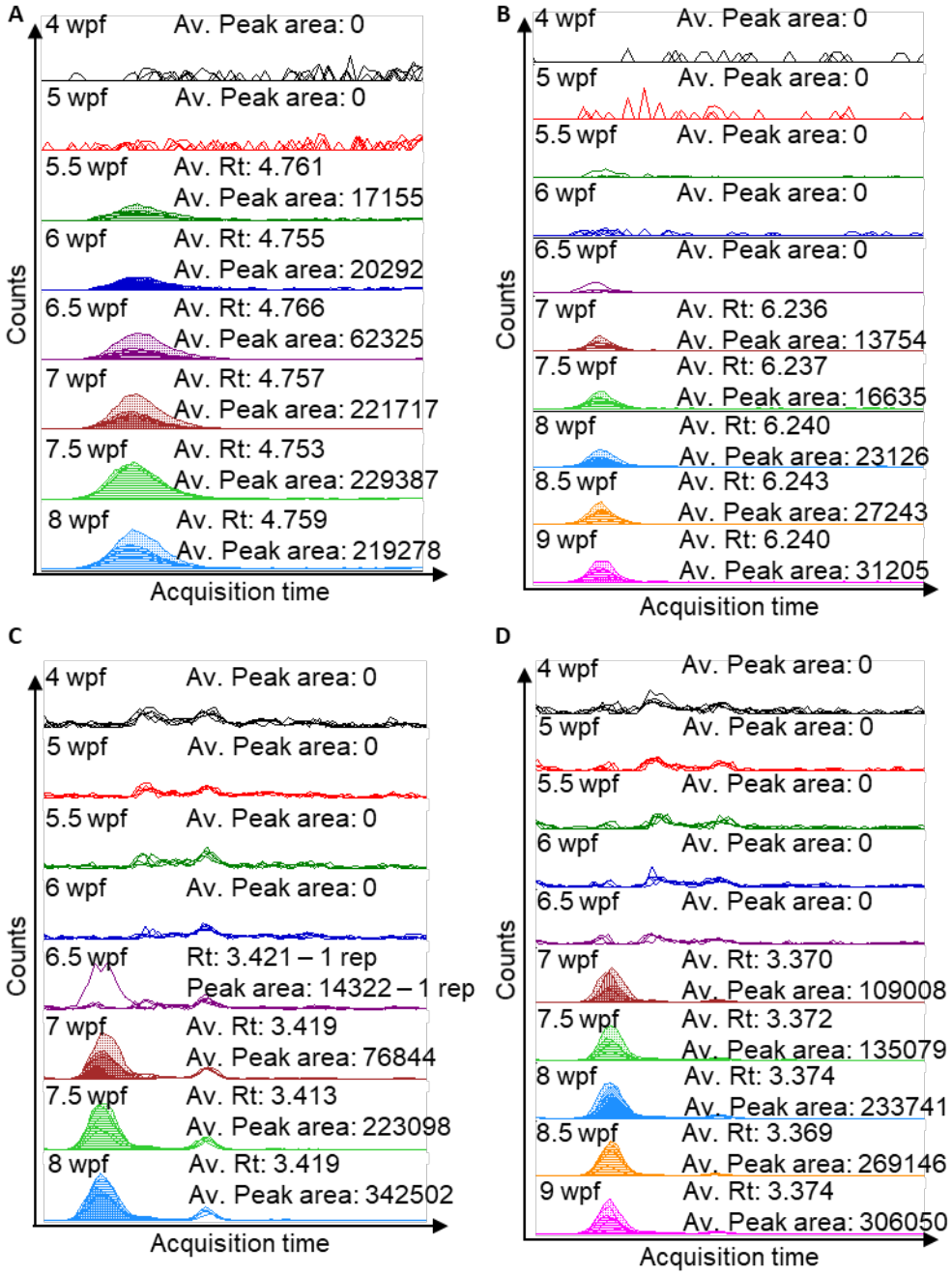


Figure 68. Extracted compound chromatograms (ECCs) of MeOH/water-extracted metabolites with high abundance in Shiraz grapes at high efficacy spray time-points. (A) and (C) show the overlaid, integrated ECC data for two example compounds from 2015/16 extracts. (B) and (D) display ECCs for the same two compounds from 2016/17 extracts.

Filtering of water-soluble NAA efficacy markers based on their occurrence in grape tissues

Another important consideration in the marker metabolite screening process was the localisation of candidate compounds within the grape berry as skin/flesh localised compounds would be the preferred choice for easy detectability in juice samples. Since the remaining 23 compounds-of-interest could all be found at reasonably high abundance in either 4 wpf or 8 wpf whole grape samples in both seasons, these two time-points were chosen for tissue localisation studies. MeOH extracts of 1 g skin/flesh and seed samples were subjected to QTOF-MS analysis in both ionisation modes as previously done with whole

grape samples. The analysis revealed that 15 of the 23 compounds were either seed-specific, had inconsistent localisation results between the two seasons, or were characterised by more than 10-fold lower ion signal intensities than the eight compounds listed in Table 33. Those eight compounds displayed the strongest signal intensities in skin/flesh tissue and were deemed the most promising candidates for further assessment and purification.

Table 33. Potential, water-soluble marker compounds with high abundance in skin/flesh of Shiraz grapes from two growing seasons.

Compound#	Av. Mass	Av. Rt (min)	Av. Signal counts	
			Skin/flesh	Seed
4 wpf samples				
1	111.0700	1.853	180,000	280,000
2	145.0530	4.233	300,000	10,000
11	320.0540	5.878	70,000	70,000
16	424.1936	5.920	70,000	20,000
8 wpf samples				
14	377.1830	5.107	50,000	500,000
15	414.1730	3.259	8,000	2,000
17	436.1010	6.266	2,000	15,000
34	384.1600	1.294	1,200,000	5,000

Robustness assessment of the top ranked potential NAA efficacy marker compounds

Structure elucidation techniques that are not based on LC-MS/MS fragmentation patterns require the purification of milligram quantities of the unknown compounds, which, depending on the compound concentration in biological extracts, can be a very difficult and time-consuming task. It was therefore decided to analyse the accumulation patterns of the top eight marker candidates in grapes from a third season in Shiraz and across two seasons in another cultivar, Cabernet Sauvignon, to concentrate subsequent identification efforts on the most promising, cultivar-independent compounds. MeOH extracts of 1 g skin/flesh samples were used for this purpose and Table 34 summarises the outcome of the QTOF-MS analysis, focussing on compound performance as high NAA efficacy markers based on positive or negative correlations between abundance patterns and efficacy rankings.

Table 34. Robustness assessment of NAA efficacy correlations for the eight highest ranked marker compound candidates. nd = not detected

Compound#	NAA efficacy correlation (positive/negative/none)					Purification target (Y/N)
	Shiraz			Cabernet Sauvignon		
	2015/16	2016/17	2018/19	2018/19	2019/20	
1	negative	negative	negative	negative	negative	Y
2	negative	negative	negative	negative	negative	Y
11	negative	negative	negative	negative	negative	Y
16	negative	negative	none	nd	nd	N
14	positive	positive	positive	positive	positive	Y
15	positive	positive	positive	nd	nd	N
17	positive	positive	positive	positive	positive	Y
34	positive	positive	positive	positive	positive	Y

Two compounds (#15 and #16) could not be detected in Cabernet Sauvignon grapes, whereas the remaining six compounds were confirmed as either positively or negatively correlated with the application-time-point-dependent efficacy of NAA treatments in this second cultivar. All six of these compounds also proved to have stable abundance patterns over three consecutive seasons in Shiraz grapes (Table 34), making them prime candidates for further identification studies.

6.12.3.3 Metabolite purification for structure elucidation by nuclear magnetic resonance (NMR) analysis

With the aim to elucidate the structure of the six putative marker compounds that had emerged from the screening process outlined above, MeOH extracts from 30 g of 4 wpf and 8 wpf skin/flesh Shiraz 2016/17 tissue were produced and containing metabolites were separated by reverse-phase HPLC, essentially replicating the HPLC-QTOF-MS run conditions used for their initial detection. One-minute fractions were collected throughout each HPLC run (25 runs per extract), pooled and tested for the six compounds-of-interest by HPLC-QTOF-MS (data not shown). It was decided to initially proceed with two compounds only, compound#2 and compound#34, as out of the six remaining target compounds, these two had the highest signal intensities, indicating that they might be reasonably abundant in grapes. Compound#2 has a negative correlation with NAA efficacy (Table 34), so could serve as a marker for optimal spray time by detecting its absence and compound#34 has a positive correlation with NAA efficacy, meaning its presence could serve to indicate the time-point for spray application when aiming to achieve maximum ripening delay.

Both compounds were further purified by two rounds of HPLC, with the first round employing an extended ACN gradient and the second round using isocratic, low percentage ACN conditions. Figure 69 shows the distribution and abundance of compound#2 as determined in round one HPLC fractions via QTOF-MS and Figure 70A provides an example of the round two purification of compound#34. Pooled fractions were assayed for compound abundance via HPLC-QTOF-MS as shown in Figure 70B.

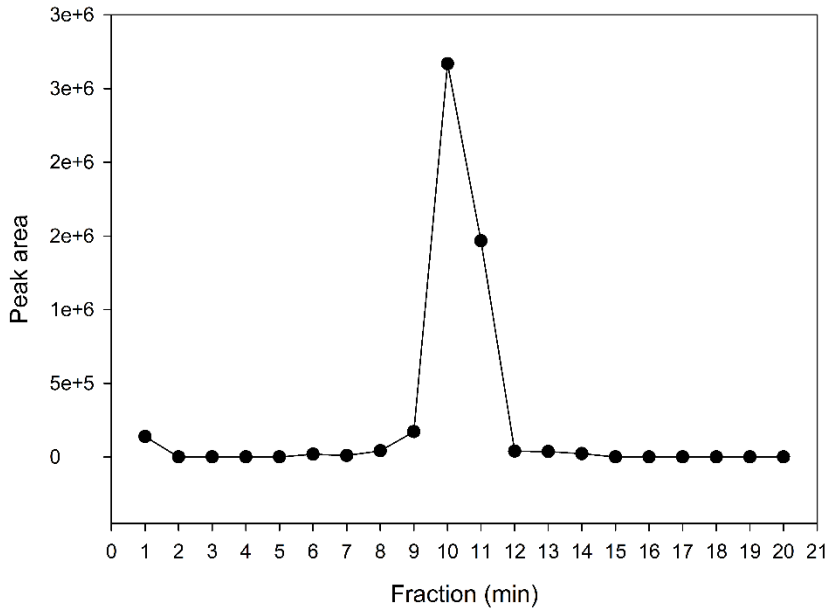


Figure 69. Abundance of compound#2 in the first round of HPLC purification. Shown is an example of the distribution of compound#2 as measured by QTOF-MS in HPLC-separated fractions of a 4 wpf MeOH extract of Shiraz berry skin/flesh using an ACN gradient.

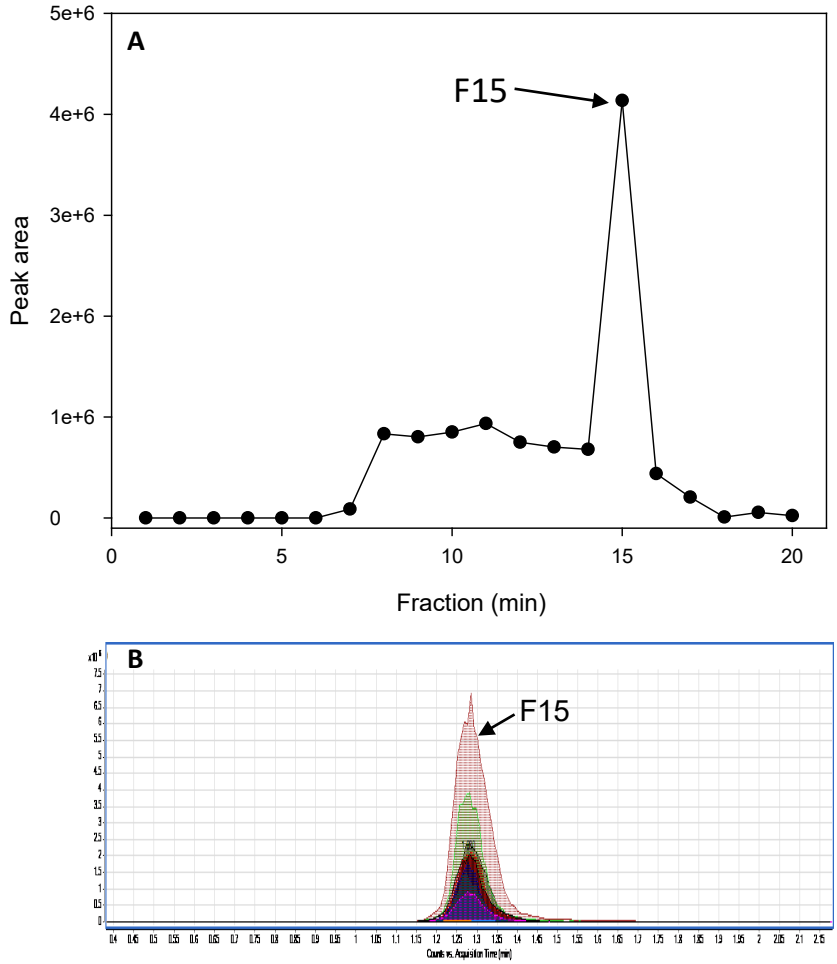


Figure 70. Abundance of compound#34 in the second round of HPLC purification. (A) Shown is an example of the distribution of compound#34 as measured by QTOF-MS in HPLC-separated fractions of an 8 wpf MeOH extract of Shiraz berry skin/flesh using isocratic 3% (v/v) ACN conditions. The overlaid QTOF-MS EIC chromatograms of the 20 tested HPLC fractions for compound#34 are shown in (B). Arrows point to fraction 15 (F15) which contained the highest concentration of compound#34.

Fractions with the highest abundance of the compounds-of-interest and a high degree of purity as judged by UV absorbance spectra and analysis of contaminants via QTOF-MS (data not shown), e.g. fraction 15 for compound#34, were dried and sent to Dr. Rob Keyzers, Victoria University of Wellington, NZ, for NMR analysis.

NMR chromatograms returned no discernible peaks apart from solvent peaks, suggesting that the analysed samples did not contain sufficient amounts of the compounds-of-interest (data not shown). Increasing the amount of extracted tissue to 100 g and the subsequent HPLC purification to a larger size, preparative column, resulted in the same, negative NMR analysis outcome.

The inability to extract sufficient quantities of the compounds-of-interest for NMR analysis from 100 g of tissue, necessitated the move to large-scale chromatographic separation of grape extracts. The chosen fractionation method of cyclic loading has successfully been used for the separation of grape material by our group (Blackford *et al.,* 2018). This method involves the passing of aqueous dilutions of a crude organic extract through a poly(styrene-divinylbenzene) (PSDVB) resin or other reverse phase matrix, thereby allowing non-polar and amphiphilic compounds-of-interest to bind to the column matrix to be

subsequently eluted with aqueous/organic solvents of increasing organic content. The result is a fractionation of the crude extract by polarity of the compounds contained in that extract. Figure 71 shows the sample purity that was achieved for compound#2 after 1 kg of Shiraz grape skin/flesh was extracted with MeOH, loaded onto PSDVB resin, eluted with 15% (v/v) aqueous ACN and passed through the two rounds of HPLC purification described above. Apart from the solvent peak observed between 6-6.5 min, absorbance profiles at 210 nm, 254 nm and 280 nm revealed only minor contamination of the purified compound#2 fraction, which was deemed suitable for NMR analysis.

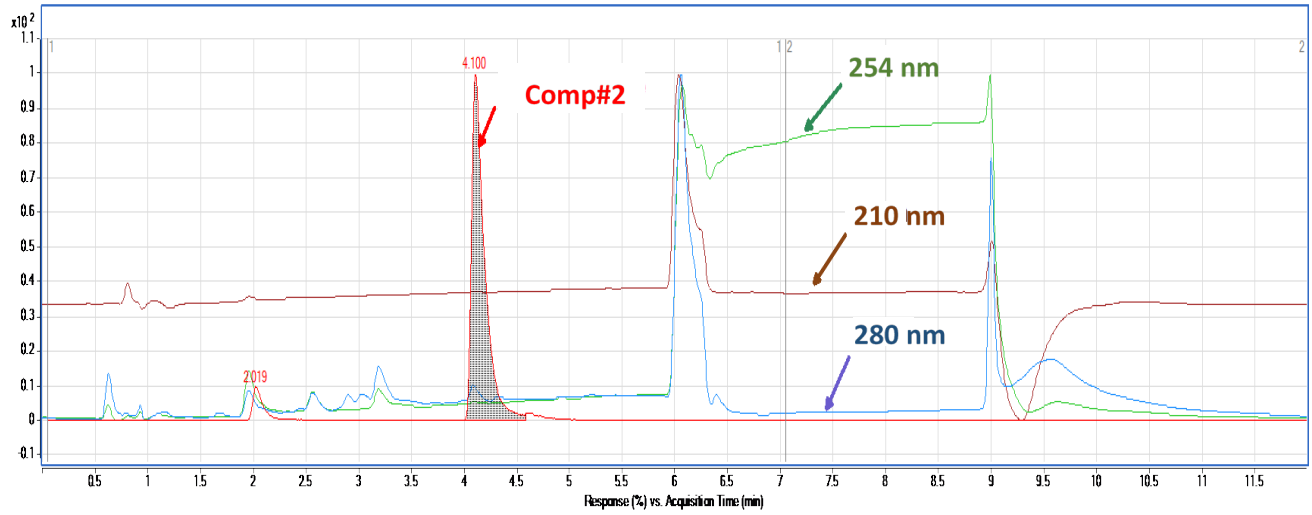


Figure 71. Purity assessment of NMR analysis sample of compound#2 after PSDVB and HPLC separation. The red trace shows the EIC of compound#2 as measured by QTOF-MS, the green, brown and blue traces display the absorbance spectra at the indicated wavelengths.

For both, compound#2 and compound#34, as well as for 14 other compounds contained in the core group of 48 compounds-of-interest (Table 32) and co-purified with compounds#2 and #34, all attempts to obtain enough pure material for structural identification via NMR from 1-2 kg of grape tissue failed. This was despite the employment of different stationary matrices (styrene-divinylbenzene adsorbent resin, modified silica gels) and elution solvents (ACN, ethyl acetate, EtOH, acetone) and profiles.

One last attempt to identify one of the compounds-of-interest was directed at compound#2. Since this compound is a small molecule with strong indications from METLIN library searches based on accurate mass and fragmentation spectra (see Figure 72) as well as further structural prediction studies using MSC (data not shown) that it is an indole or oxyquinoline compound, we decided to order available commercial standards for five of the compounds that matched its accurate mass and suggested structural features: 3-formyl-indole, 4-formyl-indole, indole-3-carboxaldehyde, 8-hydroxyquinoline (= oxyquinoline) and 4-hydroxyquinoline. Indole-3-carboxaldehyde, 3-formyl-indole, 4-formyl-indole and 8-hydroxyquinoline could be excluded as matches for compound#2 as they differed in retention time, fragmentation pattern, or both, exemplarily shown for 4-formyl-indole in Figure 73.

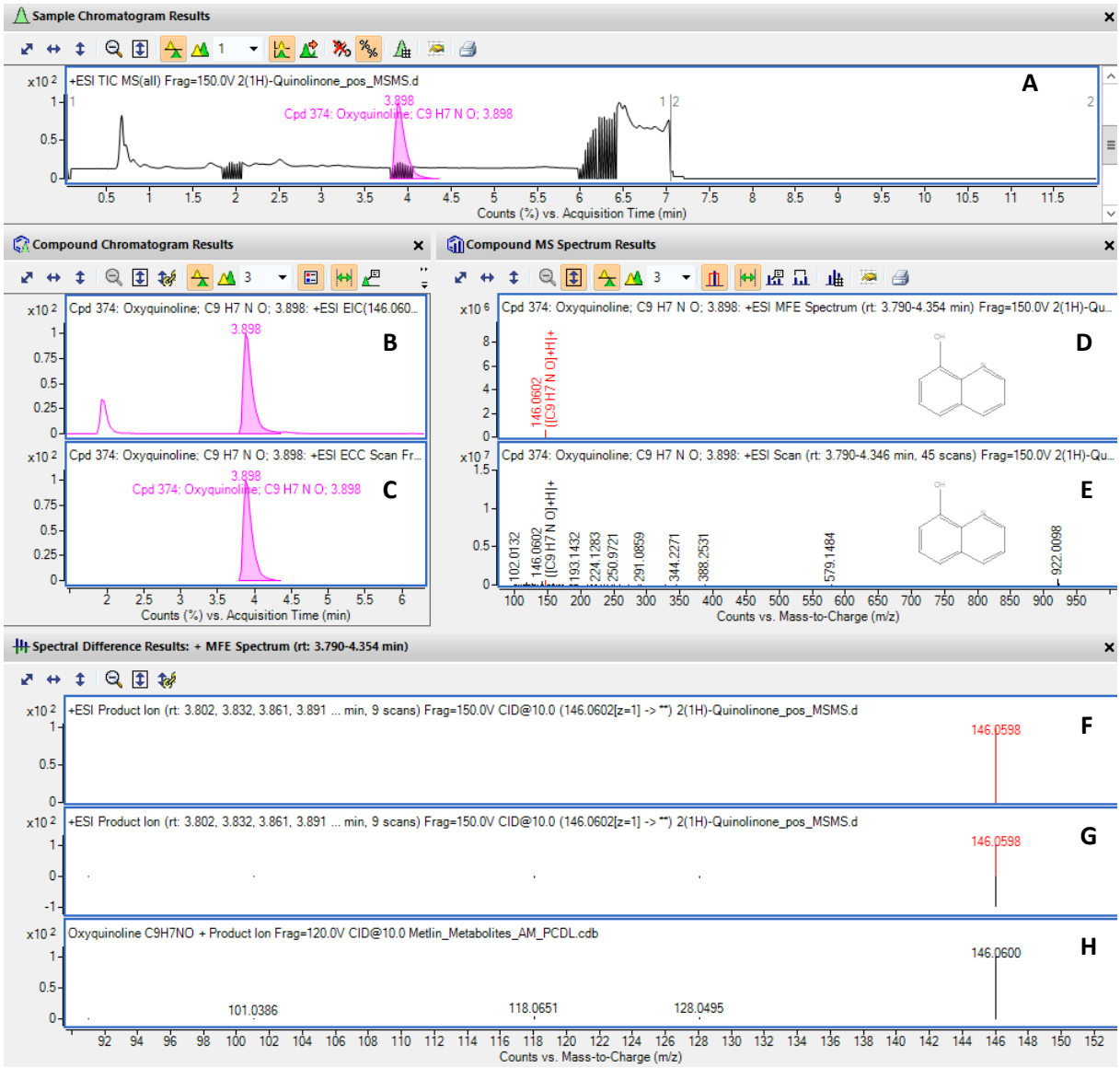


Figure 72. Compound#2 displays characteristics of an oxyquinoline-like compound. Compound#2 (A) total ion chromatogram (TIC), (B) EIC, (C) ECC, (D) extracted MFE spectrum, (E) MS scan spectrum and (F) MS/MS spectrum. (G) shows the mirror spectrum of compound#2 (red) and oxyquinoline (black) and (H) represents the METLIN library oxyquinoline spectrum.

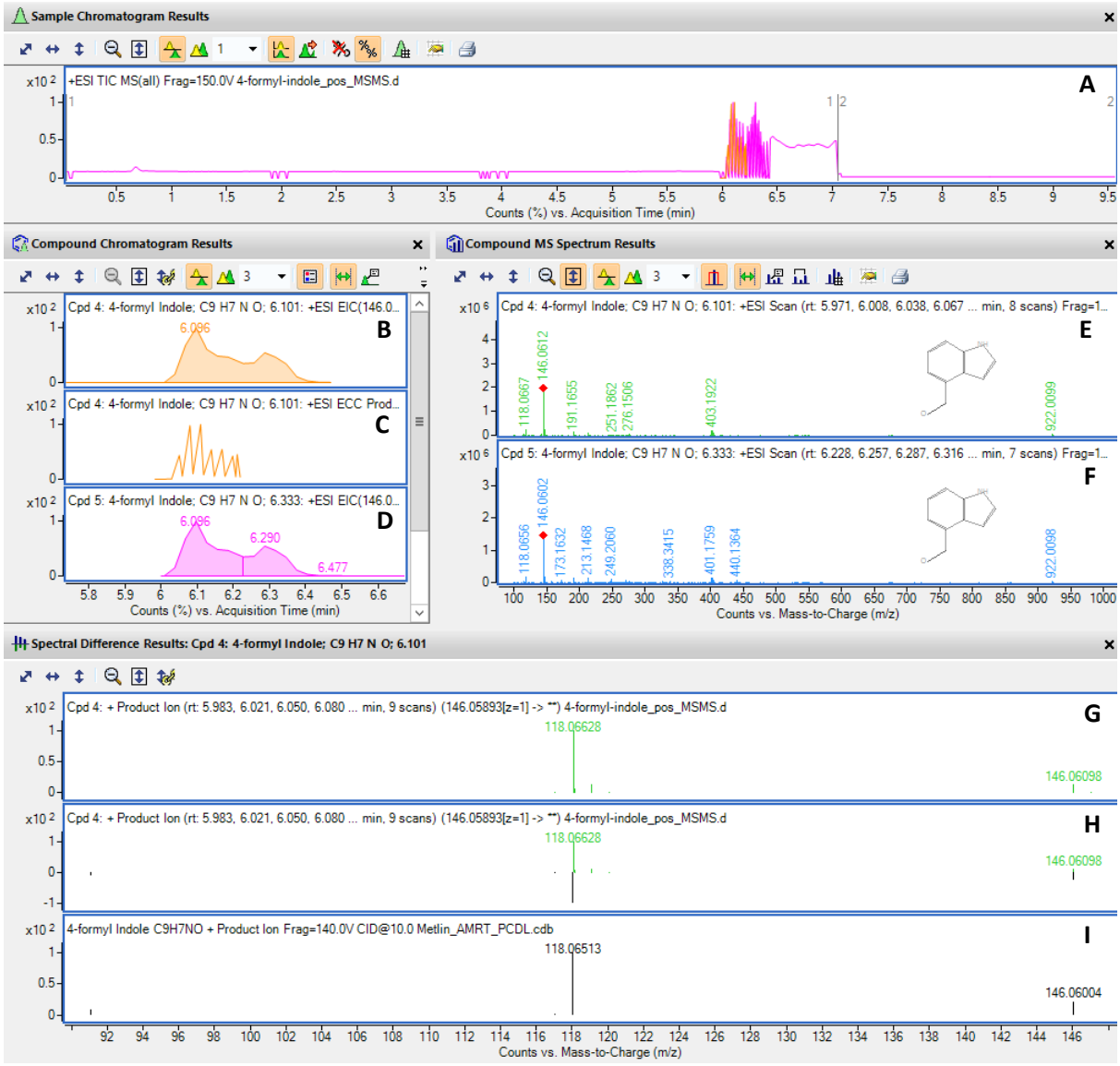


Figure 73. Example of a compound with the same accurate mass, but different chromatography and fragmentation as compound#2. (A) TIC, (B) EIC of first peak, (C) ECC of first peak, (D) ECC of second peak, (E) MS scan spectrum of first peak, (F) MS scan spectrum of second peak and (G) MS/MS spectrum of 4-formyl-indole. (H) shows the mirror spectrum of the injected 4-formyl-indole standard (green) and the METLIN library 4-formyl-indole (black) and (I) represents the METLIN library 4-formyl-indole spectrum.

4-Hydroxyquinoline on the other hand matched compound#2 in accurate mass, retention time and MS/MS fragmentation (Figure 74) and represents the first, and only, successful resolution of the identity of a compound-of-interest in this project. The presence of an aromatic ring structure and a hydroxyl group in this compound might lend themselves to the development of a simple detection system. However, this, as well as the identification of further potential NAA efficacy marker compounds, would have to be pursued in a future project.

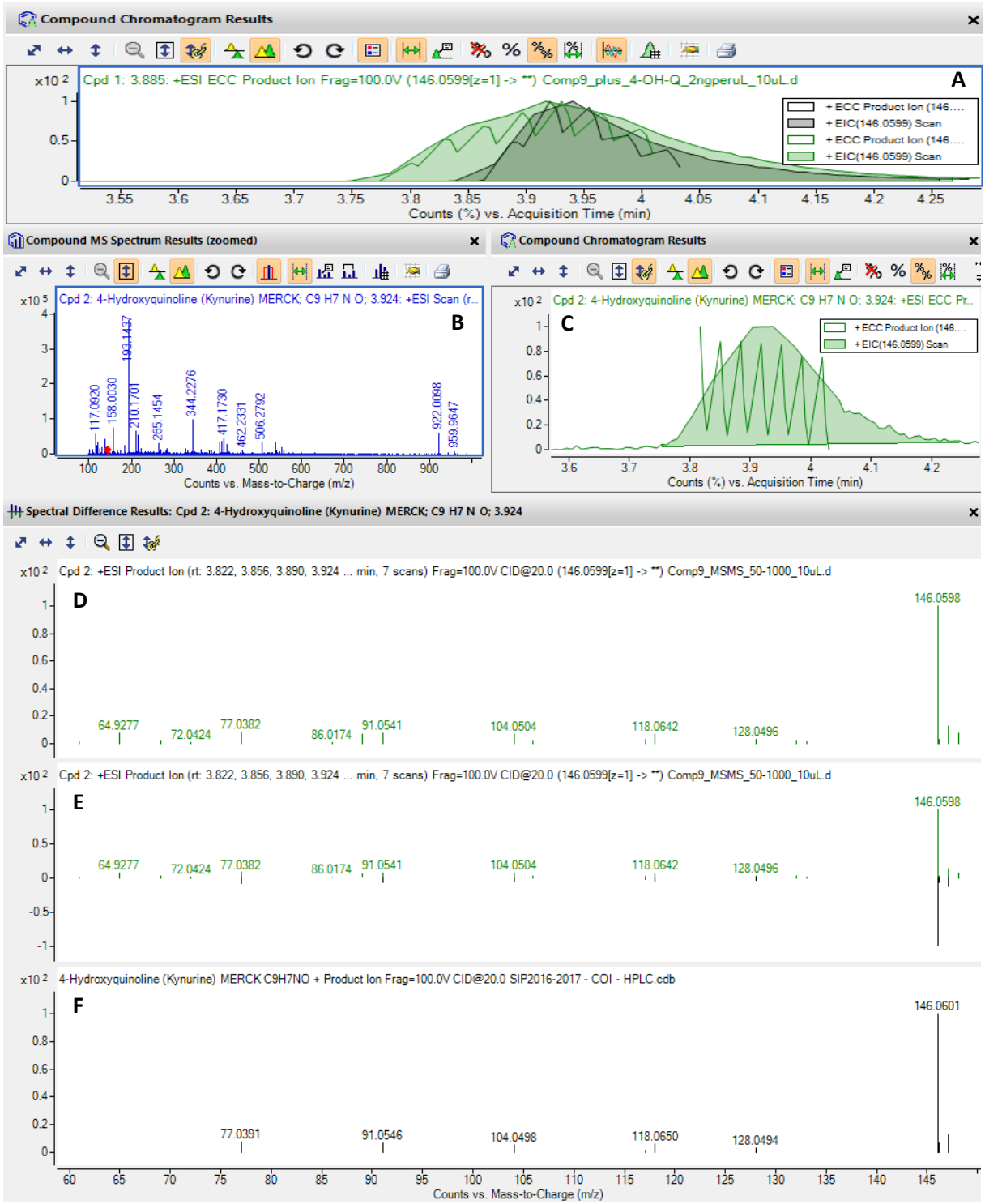


Figure 74. 4-Hydroxyquinoline is a match for compound#2. Compound#2 and 4-hydroxyquinoline co-elute when injected as a mixture. (B) MS scan spectrum (C) EIC and ECC and (D) MS/MS spectrum of 4-hydroxyquinoline. (E) shows the mirror spectrum of compound#2 (green) and the injected 4-hydroxyquinoline standard (black) and (F) represents the injected 4-hydroxyquinoline standard spectrum.

A better understanding of the relationship between sugar accumulation, hang time and flavour/aroma development

The next three sections relate to experiments involving girdling that were part of the previous project that were not reported in the previous final report. This was due to the time needed to analyse data and the timing of harvest being close to the end of the project. Consequently, the first output/activity in the current project was directed at completing the detailed analysis of the samples produced by these experiments, which are reported here. The aim of this work was to better understand the relationship between berry sugar content and wine flavour and aroma, which has been disrupted somewhat by climate change. Understanding this nexus is valuable in our efforts to develop methods to delay the timing of harvest without causing deleterious effects on wine style/quality. An important question to be answered is: whether the accumulation of flavour/aroma volatiles and their precursors occur at lower sugar concentrations (berry TSS) if the hang time is extended through reducing the rate of sugar accumulation.

6.13 Peduncle-girdling of Shiraz (*Vitis vinifera* L.) bunches alters amino acid and hormone content of grapes and affects wine volatile compounds depending on berry sugar levels

6.13.1 Introduction

Numerous studies have shown that the increase in growing season temperatures is affecting grapevine vegetative and reproductive development, which has negative implications for grape, and hence wine, quality attributes (Coombe and Iland 2004; Jones *et al.,* 2005; Mira de Orduña 2010; Varela *et al.,* 2015).

A greater understanding of the contributions of sugar accumulation and maturation time to the development of wine flavour and aroma is essential to predicting and combating the effects of climate on berry and wine composition. A study by Boss *et al.* (2014), assessing the influence of berry sugar accumulation and harvest time on the volatile profile of Riesling wine, has shown that positive influence varietal compounds were mostly correlated with berry sugar content while the reduction of compounds with potential negative impact on wine flavour and aroma was harvest time- rather than sugar-dependent. Thus, the negative effect on fruit and wine composition commonly attributed to grapes harvested at low to moderate sugar levels (Varela *et al.,* 2015) is more related to hang time than berry sugar content. The delay of grape berry 'sugar-ripeness' is therefore a promising strategy to achieve the production of wines with moderate alcohol levels and desired flavour/aroma profiles.

Various pruning methods have been successfully used to significantly delay the accumulation of sugar in a range of grapevine cultivars. A recent study by Pineau *et al.* (2017) investigated if the delay of sugar accumulation by canopy trimming resulted in changes to the sensory properties of wine made from *Vitis vinifera* L. cv. Pinot Noir berries of different sugar content, but identical 'hang time'. Although the Brix range was small, wines made from fruit with 'moderate (22.1-22.5 °Brix) and high (23.2-23.9 °Brix) sugar levels' were indistinguishable with regard to their sensory attributes.

Delaying the accumulation of sugar in grape berries has the potential to shift later stages of ripening into the cooler part of the season, ameliorating excessive sugar accumulation and increasing 'hang time' on the vine to help decrease undesired wine volatiles or their precursors.

This experiment was aimed at improving our understanding of the influence of sugar accumulation on the volatile composition of wines by peduncle-girdling of Shiraz bunches at three different times. The separation of berry sugar accumulation from secondary metabolism was intended to provide additional insights into the dependence of wine volatile and non-volatile compound production on 'sugar-ripeness'.

6.13.2 Materials and methods

Grapes and girdling

Bunches from *Vitis vinifera* L. cv. Shiraz vines grown at Willunga, South Australia were peduncle-girdled on three dates post-veraison (14 Jan 2010 (Girdle 1), 20 Jan (Girdle 2), 29 Jan (Girdle 3)) by removing a 1-2 mm wide section of the phloem with a razor blade.

Girdling was performed in one of three adjacent rows in triplicate, with 15 bunches from three adjacent vines constituting one replicate. The same number of un-girdled bunches were used as Controls.

At the following time-points after girdling, berries (three per bunch, 45 per replicate) were sampled, weighed, de-seeded, frozen in liquid nitrogen and stored at -80°C: 0 (Control only), 6, 15, 21, 29 days (Girdle 1); 0 (Control only), 9, 15, 23 days (Girdle 2); 6, 14 days (Girdle 3). At the time of commercial harvest (22 Feb), all girdled and Control bunches were removed from the vines, berries were removed from the rachis, combined according to treatment and replicate, frozen whole and used for TSS and anthocyanin analysis.

LC-ESI-MS/MS analysis of plant hormones

Powdered berry tissue (100 mg) was used to extract and quantify the following plant hormones using previously published LC-ESI-MS/MS methods: indole-3-acetic acid (IAA) and IAA-aspartic acid (IAA-Asp) (Böttcher *et al.,* 2011, 2012b), *trans*-zeatin (*t*Z) and N^6-(Δ^2-isopentenyl)-adenine (iP) (Böttcher *et al.,* 2015a), abscisic acid (ABA) and selected ABA metabolites (Speirs *et al.,* 2013).

qRT-PCR

The gene-specific primers for the following genes analysed in this study were published previously: *ACT2* (reference gene; Böttcher *et al.,* 2011a); ethylene-related genes (Böttcher *et al.,* 2013c), ABA-related genes (Wheeler *et al.,* 2009), auxin-related genes (Böttcher *et al.,* 2011a, 2013b) and cytokinin-related genes (Böttcher *et al.,* 2015a). The grapevine orthologues of *Arabidopsis thaliana* L. proline biosynthesis, degradation and transport genes (Table 35), obtained from were identified by BLASTN searches of the nucleotide NCBI (http://www.ncbi.nlm.nih.gov/) and CRIBI (http://genomes.cribi.unipd.it/grape) databases.

Table 35. Gene-specific primer pairs for proline-relates genes used for qRT-PCR analyses.

Gene	NCBI reference sequence	CRIBI (V2) locus ID
VviP5CS1	XM_002273220	VIT_208s0007g01070
VviP5CS2	NM_001281205	VIT_215s0024g00720
VviP5CR	XM_003632680	VIT_208s0007g05600
VviPDH	XM_002282733	VIT_214s0083g00520
VviP5CDH	XM_002273533	VIT_217s0000g05790
VviProt	XM_002280181	VIT_213s0019g03220

Fermentation conditions and headspace volatile analysis

Sub-samples (25 g) of the ground Shiraz harvest samples were fermented and analysed for headspace volatiles as described by Boss *et al.* (2014).

Data analysis

ANOVA was performed for the Shiraz berry gene expression and proline data, followed by Duncan's post hoc test for Girdle 1 samples and by Fisher's Least Significant Difference (LSD) post hoc test for developmental samples. Grape amino acid and wine volatile data were analysed by ANOVA testing for any significant row effect. Analytes with a significant row effect were removed from further analyses as this may confound any girdling effect. For the remaining compounds, the Controls were combined and ANOVA performed, testing for any significant effect of girdling, followed by Duncan's post hoc test. Correlation analyses were performed using the Regression Wizard function of SigmaPlot (ver. 12.5; Synstat Software, San Jose, CA).

6.13.3 Results and Discussion

Effect of peduncle-girdling on grape berry development

The peduncle-girdling of Shiraz bunches occurred at three post-veraison stages of berry development. Girdle 1 (14 Jan) was performed when the berries had reached an average TSS level of 10.3 °Brix and was followed by the monitoring of TSS levels, berry weight and anthocyanin accumulation until harvest, 39 days after the girdling event (Figure 75 a,d,g). During this time, Control berries, taken from non-girdled bunches of a comparable developmental stage at the time of girdling, showed a significant increase in TSS levels at every sampling time point, reaching 27.1 °Brix at harvest, with a maximum average berry weight of 1.6 g at 21 days post girdle (dpg). The TSS levels of berries from girdled bunches were significantly lower than those of Control berries at every sampling time-point with a significant increase in TSS levels occurring between 15-29 dpg. This TSS increase could be attributed to a reduction in berry weight during the same time period as shown by a strong negative correlation between TSS and berry weight ($r = -0.9874$; $n = 6$; $p < 0.001$). At harvest, Girdle 1 berries reached 18.5 °Brix and an average weight of 0.5 g. Anthocyanin concentration increased in Control and Girdle 1 berries until 29 dpg but was significantly lower in Girdle 1 berries from 15 dpg onwards. Again, a negative correlation between berry weight and anthocyanins ($r = -0.9967$; $n = 6$; $p < 0.02$) in Girdle 1 berries suggested berry shrivel, rather than anthocyanin production, as the main cause for the observed rise in concentration. At the last sampling time-point, the anthocyanin concentration in Control berries plateaued, but it decreased in Girdle 1 berries.

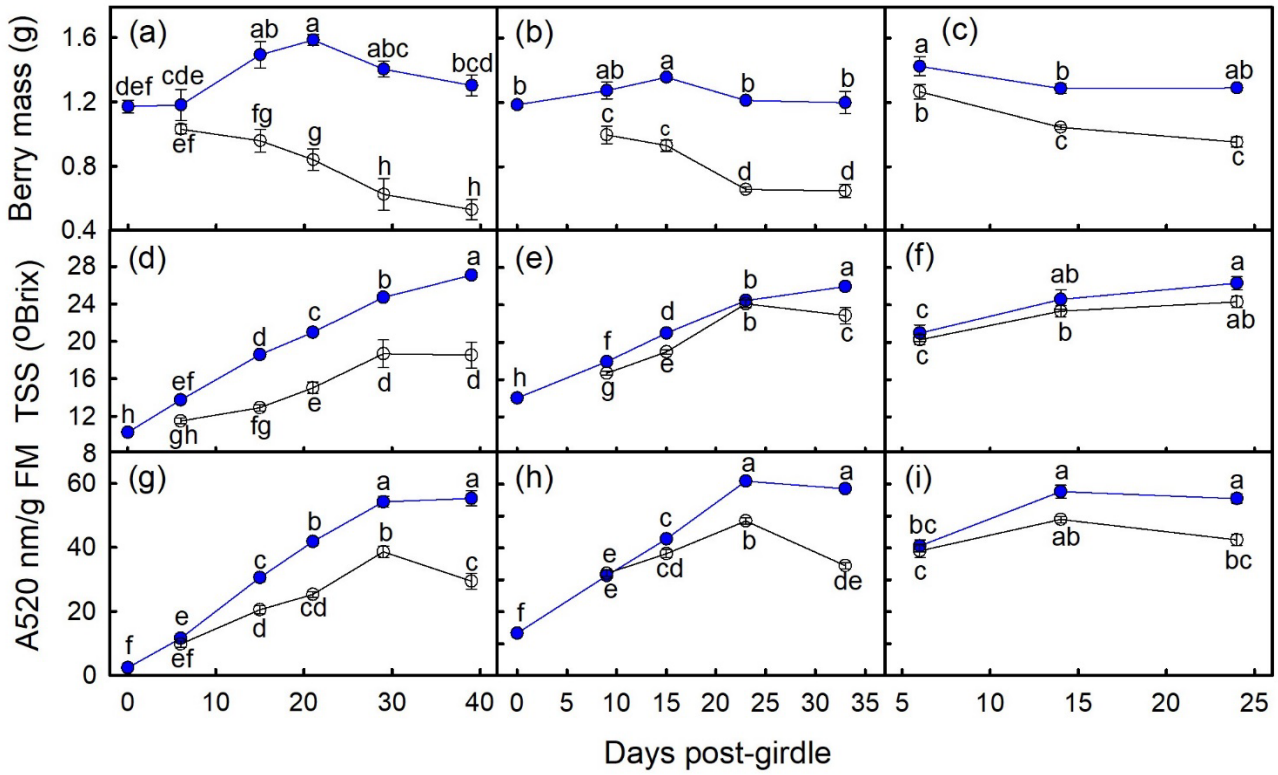


Figure 75. (a-c) Weight, (d-f) TSS and (g-i) anthocyanin (A520 nm) accumulation in developing Shiraz Control (●) and peduncle-girdled (○) berries, girdled at (a, d, g) 14 Jan (Girdle 1), (b, e, h) 20 Jan (Girdle 2) and (c, f, i) 29 Jan (Girdle 3). Values represent means ± SE (*n* = 3) and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test. FM, fresh weight.

At the time of Girdle 2 (20 Jan), berries had an average TSS level of 14 °Brix and were analysed for TSS, weight and anthocyanin content at regular intervals until harvest at 33 dpg (Figure 75 b,e,h). Similar to Girdle 1, Control berries were characterised by a steady increase in TSS levels, peaking at 26 °Brix, and small changes in berry weight, with a maximum of 1.4 g at 15 dpg. Compared with Girdle 1, the TSS difference between Control and Girdle 2 berries was less pronounced, but TSS levels of Girdle 2 berries were significantly lower than those of Control berries at all but one sampling time point and reached 22.9 °Brix at harvest. As with Girdle 1, a significant decrease in average berry weight occurred between 15-21 dpg, and at harvest the average berry weight was 0.6 g. Berry weights of girdled bunches were lower than Control berry weights at all sampling time points. A significant negative correlation between weight and TSS for Girdle 2 berries ($r = -0.9883$; $n = 5$; $p < 0.01$) identified berry shrivel as the likely cause for the TSS increase. Anthocyanin concentration decreased in Girdle 2 berries at harvest.

Girdle 3 (29 Jan) was performed at an average TSS level of 18 °Brix and Control and Girdle 3 berries were sampled at 6, 14 and 24 dpg for TSS, weight and anthocyanin measurements (Figure 75 c,f,i). The average berry weight was consistently lower in Girdle 3 berries than in Control berries, but there was no significant difference in TSS levels between Girdle 3 and Control berries. A small TSS increase was observed for both treatments between 6 and 14 dpg and harvest TSS were 26.3 °Brix (Control) and 24.3 °Brix (Girdle 3). As for the earlier girdling time points, berry shrivel was the likely cause for the observed increase in TSS and anthocyanin levels in Girdle 3 berries.

Phloem disruption prevented sugar accumulation since any observed TSS increase can be attributable to a simultaneous reduction in berry weight (Figure 75). Berry shrinkage due to the girdling treatment and was most likely caused by phloem disruption coupled with a reduced xylem flow in post-veraison grapes (Creasy and Lombard 1993), which led to reduce water content as a result of water loss via transpiration. The reduced accumulation of anthocyanins in berries derived from peduncle-girdled Shiraz bunches has also been described previously (Hall *et al.,* 2010). However, despite a reported correlation between grape berry sugar uptake and anthocyanin production (Rogiers and Holzapfel 2015), a definite cause for the poor colour development in berries with reduced phloem connectivity is yet to be determined. Similarly, the degradation of anthocyanins detected at harvest of Girdle 1 and 2 berries (Figure 75g,h) has previously been described during the late stages of Shiraz berry shrivel (Shivashankara *et al.,* 2013, Rogiers and Holzapfel 2015) but no convincing explanation of this phenomenon has been advanced. However, membrane damage and cell death could be contributing factors (Krasnow *et al.,* 2008).

Wine volatiles and berry amino acids affected by peduncle-girdling

In order to assess the effect of restricting sugar uptake at different berry sugar base levels on wine volatiles, 50 mL model wine batches adjusted for their glucose/fructose concentration (Table 36) were produced and analysed for their volatile compounds using SPME GC-MS.

Table 36. Grape chemical parameters of Control and Girdle Shiraz berries used for fermentation. All values represent the mean ± SE of n = 3 biological replicates.

Girdle time point	Treatment	YAN (mg N L^{-1})	Glucose + fructose (g L^{-1})
Girdle 1 (14 Jan)	Control	214 ± 33	198 ± 13
	Girdle	255 ± 37	148 ± 9
Girdle 2 (20 Jan)	Control	189 ± 12	210 ± 8
	Girdle	215 ± 4	183 ± 7
Girdle 3 (29 Jan)	Control	212 ± 14	222 ± 4
	Girdle	201 ± 8	193 ± 1

Fifty eight out of a total of 151 quantified compounds were significantly different ($p < 0.05$) in abundance in the headspace of the wines made from Control fruit and Girdle fruit (Table 37). Forty three of these compounds were more abundant in one or more of the Girdle wines than the Control wines, 20 were esters. Nine of these were acetate esters, consisting of ethyl acetate and eight higher alcohol acetates (HAAs) and the eight ethyl esters in this group were a mixture of straight chain fatty acid, branched chain fatty acid and alpha-hydroxy acid esters. There were nine higher alcohols, four C_{13}-norisoprenoids, four acetals and aldehydes, three monoterpenes and single carboxylic acids and alkenes. Early girdling had the biggest effect, with 34 out of the 43 compounds more abundant in Girdle 1, and in the case of 22 of these compounds also Girdle 2, wines compared with the Control. Nine compounds showed an increased concentration in Girdle 2, but not Girdle 1, wines when compared with the Control, with six of these compounds also present in significantly higher concentrations in Girdle 3 wines compared with the Control wines. The remaining 15 out of the 58 identified compounds with significant differences between Girdle and Control wines were less abundant in one or more of the Girdle wines than the Control wines. As described for the compounds with increased concentration in Girdle wines, these were mainly esters; 2-phenylethyl esters accounted for four out of the nine compounds in this group. Most of the changes were observed in Girdle 1 wines, where 13 of the compounds were found to be less abundant than in

Control wines. For five of the 13 compounds this was also true for Girdle 2 wines and two compounds, ethyl 2-furoate and 3-(methylthio)-1-propanol, were reduced in abundance in all three Girdle wines. The concentration of octanoic acid was significantly lower in Girdle 2 wines only, when compared with the Control and for geranyl acetate this was the case in Girdle 3 wines.

Table 37. Volatile compounds found to be significantly different in the headspace of the wines produced from Shiraz berries of bunches that were peduncle-girdled at three different time points/Brix levels (Girdle 1: 10.3 °Brix, Girdle 2: 14.0 °Brix, Girdle 3: 17.9 °Brix) and Control berries.

Compound	Girdle 1	Girdle 2	Girdle 3	Control
Higher in one or more girdled sample(s) than the Control				
Ethyl acetate†	5143.3a	4174.3b	3385.7c	3498.4bc
Ethyl propanoate‡	45.8a	43.9ab	37.9ab	35.4b
Ethyl 2-methylpropanoate‡	18.7b	23.7a	19.8b	19.6b
Propyl acetate	149.5a	78.0b	51.4b	47.6b
2,4,5-Trimethyl-1,3-dioxolane	19.2ab	26.6a	26.1a	10.4b
2-Methylpropyl acetate†	132.7a	103.7b	77.5c	78.9c
2,4,6-Trimethyl-1,3-dioxolane	9.4ab	14.0a	12.5a	4.8b
Ethyl butanoate†	690.8a	628.8ab	529.7c	584.9bc
1-Propanol†	653.3a	460.0b	316.2c	275.8c
Butyl acetate	24.4a	17.7b	13.7b	12.1b
3-Methylbutyl acetate†	45.3a	35.0b	26.6c	28.4bc
Ocimene	3.2ab	4.1a	3.4a	2.2b
2-Isobutyl-4,5-dimethyl-1,3-dioxolane	43.7ab	57.2a	53.1a	32.5b
Hexyl acetate†/‡	2549.5a	1746.3b	1331.3c	1435.7c
Ethyl 3-hexenoate‡	28.8a	23.5a	13.8b	8.9b
(*E*)-3-Hexenyl acetate	47.2a	28.3bc	22.0c	29.2b
(*Z*)-3-Hexenyl acetate†/‡	212.2a	105.4b	50.8c	24.8c
Propyl hexanoate	31.6a	20.1b	14.2c	12.3c
(*E*)-2-Heptenal	19.1a	11.7ab	9.6b	5.6b
Ethyl heptanoate‡	21.1b	28.9a	19.9b	17.6b
Ethyl lactate	23.3a	15.8b	11.4b	9.8b
Ethyl 2-hexenoate‡	24.2a	23.3a	16.2b	12.6b
1-Hexanol‡	1995.2a	1964.7a	1614.7b	1594.8b
3-Ethoxy-1-propanol‡	65.9a	60.4a	40.3b	38.5b
Heptyl acetate‡	18.3a	16.4a	7.5b	5.9b
(*Z*)-3-Hexenol‡	101.8a	76.6b	32.1c	15.1c
1-Octen-3-ol	21.3a	16.8ab	7.4bc	5.4c
1-Heptanol	11.5a	11.8a	6.8b	6.6b
Decanal	15.1a	8.3b	6.5b	7.9b
1-Butyl cyclohexene	9.7a	10.7a	6.2b	5.5b
Benzaldehyde	8.2ab	9.3a	7.5b	7.2b
Propyl octanoate	18.2a	13.7b	10.4c	10.5c
Vitispirane I and II	18.7b	30.9a	28.5a	9.2b
Linalool†/‡	12.9a	12.7a	10.0ab	8.5b
Propanoic acid	31.8a	30.9ab	25.4bc	21.3c
1-Octanol‡	92.2a	95.0a	84.8ab	70.4b
β-Cyclocitral	3.9a	3.7a	2.8b	2.4b
1-Nonanol	87.0a	96.1a	67.8b	70.7b
Diethyl succinate‡	179.8a	175.9a	113.3b	128.7b
Propyl decanoate	8.3a	6.7ab	4.5b	4.7b
β-Damascenone†	71.7ab	89.0a	82.8a	50.8b
Benzyl alcohol†	27.2a	30.1a	17.4b	13.9b
β-Ionone	11.3a	8.2b	7.6b	8.8b

Higher in Control than in one or more girdled sample(s)				
3-Methylbutanol†	623.0b	693.0a	686.5ab	715.9a
Ethyl 3-(methylthio)propanoate	2.1b	3.3a	2.7ab	3.7a
2-Undecanone	13.1b	16.7ab	18.0a	19.9a
Ethyl 2-furoate‡	6.0b	7.2b	8.4b	11.7a
2-Methylbutanoic acid	120.4b	208.7a	204.4a	217.5a
Ethyl 9-decenoate	110.2c	173.5b	206.0ab	247.8a
3-(Methylthio)-1-propanol†	37.3b	54.6b	55.2c	81.8a
Geranyl acetate	8.1ab	7.8ab	7.4b	10.0a
β-Citronellol	18.1b	25.1a	25.5a	27.7a
Ethyl phenyl acetate	43.2c	68.7b	75.7ab	83.3a
2-Phenylethyl propanoate	9.5b	15.6ab	17.6a	20.9a
2-Phenylethyl butanoate†	15.1b	20.3b	21.1ab	28.5a
Octanoic acid	2267.9ab	2031.3b	2239.5ab	2661.6a
2-Phenylethyl hexanoate	97.3b	125.7b	150.0ab	198.3a
2-Phenylethyl octanoate	28.6b	39.5ab	41.2ab	51.4a

Values are the mean of n = 3 biological replicates (Girdle 1-3) or n = 9 biological replicates (Control) of the areas under the peaks of a selected ion relative to the relevant internal standard.†Increased abundance in wines made from NAA-delayed fruit (Böttcher et al., 2010, 2012a, Davies et al., 2015).‡Decreased abundance in wines made from NAA-delayed fruit (Böttcher et al., 2010, 2012a, Davies et al., 2015).

Peduncle-girdling at all three post-veraison stages caused changes in the volatiles of wines made from the corresponding fruit. However, there was a strong association between the berry sugar level at the time of girdling and the impact of the girdling treatment on the wine volatile profile (Table 37), with 47, 38 and 10 compounds significantly different ($p<0.05$) in concentration in Girdle 1, 2 and 3 wines, respectively, when compared to Control wines. The girdling-induced berry water loss was unlikely to be a major influence on the differences in volatile compounds detected between wines made from Girdle and Control berries. Šuklje *et al.* (2016) reported an overall decrease in ethyl esters and HAAs as a result of Shiraz berry shrivelling, whereas these two compound groups accounted for most of the increased volatile compounds in Girdle wines (Table 37). In addition, the abundance of monoterpenes linalool and ocimene was increased in Girdle wines, while terpenes were found to be unaltered in wines made from shrivelled berries (Table 37; Šuklje *et al.,* 2016).

The 58 wine volatiles identified as being affected by girdling (Table 37) included yeast-derived compounds such as esters and grape-derived compounds such as terpenoids and C13-norisoprenoids (Robinson *et al.,* 2014), suggesting that peduncle-girdling induced changes in the grape composition that not only affected grape-derived wine volatiles, but also influenced the production of wine volatiles during the fermentation process.

The increased abundance of the C13-norisoprenoids, β–ionone in Girdle 1, β-cyclocitral in Girdle 1 and 2 and vitispirane and β–damascenone in Girdle 2 and 3 wines, could indicate a girdling-induced stimulation of carotenoid breakdown (Baumes *et al.,* 2002). This may be due to an increased activity of carotenoid cleavage enzymes in Girdle berries (Mathieu *et al.,* 2005), caused by modifications to the cellular environment favouring oxidative reactions. A further indication for a changed redox balance in grapes impacted by girdling was the elevated concentration of several alcohols, aldehydes (Table 37; 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, (Z)-3-hexenol, (E)-2-heptenal) and corresponding acetate esters (Table 37; hexyl acetate, (E)-3-hexenyl acetate, (Z)-3-hexenyl acetate, heptyl acetate) derived from lipid oxidation (Frankel 1983), with a high representation of C6-compounds originating from the lipoxygenase pathway (Schwab *et al.,* 2008). The observed changes to lipid oxidation were confined to the first two girdling treatments, where berry shrivelling was most severe, and might therefore be related to the

mixing of berry cell contents. Alternatively, the accumulation of C6-compounds in wines made from Girdle 1 and 2 berries could be the result of a wounding response to the girdling treatment. In support of this, the wound activation of the lipoxygenase pathway and subsequent production of (E)-2-hexenal has previously been reported in strawberry (Myung *et al.,* 2006). Wound stress also provides a possible explanation for the increased concentration of linalool in Girdle 1 and 2 wines (Table 37), which seemed to contradict the reported sugar-linked accumulation of this terpene in muscat-type grapes and Riesling (Martin *et al.,* 2012, Boss *et al.,* 2014, Zhu *et al.,* 2014), since linalool production was found to be stimulated by mechanical wounding in tomato (van Schie *et al.,* 2007). However, it is also possible that the unusual accumulation pattern of linalool was due to varietal differences, as the grape varieties in the studies cited above produce higher concentrations of monoterpenes than Shiraz berries.

The occurrence of esters as the main volatile group differentiating Girdle and Control wines prompted the analysis of the berry amino acid content of Girdle and Control harvest samples since peduncle-girdling can be expected to restrict the influx of a number of amino acids (Gholami *et al.,* 2004), which are possible precursors for ester production during fermentation by yeast (Hazelwood *et al.,* 2008, Bisson and Karpel 2010, Boss *et al.,* 2015). A total of 10 amino acids displayed a significant difference in concentration between one or more Girdle and Control berries with nine of those different in Girdle 1 and/or Girdle 2 berries only (Table 38). Six amino acids were at higher concentrations in Girdle 1 and/or Girdle 2 berries, whereas four amino acids had reduced concentrations in Girdle berries. Asparagine was the most affected amino acid with a five-fold increase in Girdle 1 berries compared with Control berries. Proline showed the largest decrease in abundance in response to girdling, with a 3.6-fold reduction in Girdle 1 versus Control berries. The proline precursor, glutamic acid, was also found at a lower concentration in Girdle samples, as was the glutamic acid- or proline-derived γ-aminobutyric acid (GABA) (Delauney and Verma 1993, Signorelli *et al.,* 2015).

Table 38. Amino acids found to be significantly different in Shiraz berries at harvest of bunches that were peduncle-girdled at three different time points/Brix levels (Girdle 1: 10.3 °Brix, Girdle 2: 14.0 °Brix, Girdle 3: 17.9 °Brix) and Control berries.

Compound	Girdle 1	Girdle 2	Girdle 3	Control
			(mg/L)	
Higher in one or more girdled sample(s) than the Control				
Hydroxyproline	11.8b	17.6a	14.5ab	11.7b
Aspartic acid	33.6a	39.8a	16.9b	19.1b
Asparagine	64.0a	36.6b	14.3b	12.9b
Glutamine	79.2a	72.0a	38.8b	45.8b
Arginine	1260.5a	1104.0ab	842.6b	763.3b
Lysine	25.1a	19.3ab	13.3b	12.6b
Higher in Control than in one or more girdled sample(s)				
Glutamic acid	40.8b	42.4b	39.6b	78.9a
Proline	269.4c	590.9b	759.5ab	976.4a
γ-Aminobutyric acid	132.4b	193.7ab	234.8a	238.7a
Methionine	7.4b	4.8b	15.1a	18.0a

Values are the mean of n = 3 biological replicates (Girdle 1-3) or n = 9 biological replicates (Control).

The main discriminating factor between Girdle and Control wines was the concentration of numerous ethyl and acetate esters (Table 37). Both types of esters are produced by yeast metabolism during the fermentation process, using alcohol and acetyl-CoA\acyl-CoA precursors mainly derived from sugars and

amino acids present in the must (Sumby *et al.,* 2010). While the increase in ethyl esters in Girdle wines could indicate an increase in fatty acid precursors (Saerens *et al.,* 2008), the higher abundance of acetate esters is consistent with a stimulating effect on yeast alcohol acetyl transferase (AAT) activity (Saerens *et al.,* 2010). A potentially higher AAT activity and the consequential increased production of HAAs in Girdle wines could be the result of changes to the amino acid composition of Girdle berries. Aspartic acid, asparagine, glutamine and arginine, comprising four out of the six amino acids with increased concentration in Girdle 1 and 2 berries (Table 38), belong to a group of amino acids that is preferentially taken up and metabolised by yeast during fermentation (Ljungdahl and Daignan-Fornier 2012). Combined with the overall unchanged levels of yeast-assimilable nitrogen in Girdle berries compared with Control berries (Table 36), this relative increase in yeast-preferred amino acids in Girdle berries equated to improved access to amino nitrogen, which has been shown to stimulate AAT expression and hence HAA production (Fujiwara *et al.,* 1999). Since the transport of aspartic acid, asparagine and glutamine in grapevine rachis and petioles has been described as mainly occurring through the xylem (Gourieroux *et al.,* 2016), the amplified accumulation in Girdle berries might have been the result of a compensatory increase in xylem flow or solute concentration in response to phloem restriction.

Overall, the observed differences in grape amino acids (Table 38) were not reflected in the variation in volatiles with potential amino acid precursors (Table 37), such as alcohols and esters derived from branched chain or aromatic amino acids (Hazelwood *et al.,* 2008). For example, the higher abundance of four 2-phenylethyl esters in Control wines was not due to a higher concentration of the 2-phenylethanol precursor, phenylalanine, in Control grapes (Tables 37, 38). Instead, the higher levels of 2-phenylethyl esters in Control wines compared with Girdle wines might reflect differences in phenylalanine metabolism and could indicate an advanced maturity stage of Control berries (Antalick *et al.,* 2015).

Girdling-induced changes to proline metabolism in grape berries

The reduced proline concentration in Girdle berries (Table 38) seemed to support the notion of phloem enabled translocation of this amino acid (Glad *et al.,* 1992; Gourieroux *et al.,* 2016). However, changes to proline metabolism within the berries are a possible alternative for the observed reduction (Stines *et al.,* 1999). To clarify this, grapevine genes belonging to the families of Δ^1-pyrroline-5-carboxylate (P5C) synthetases (P5CSs), P5C reductases (P5CRs), proline dehydrogenases (PDHs), P5C dehydrogenases (P5CDHs) and proline transporters (Prots) were identified by sequence similarity to the respective *Arabidopsis* genes.

All five of the proline-related genes were found to be expressed in grape berry tissue (data not shown). The transcript abundance of the five proline-related genes expressed in berries was analysed in conjunction with proline accumulation at each sampling time point, excluding harvest, after the Girdle 1 event in berries from peduncle-girdled and Control bunches. In Girdle 1 berries, proline concentrations remained unchanged from the time of girdling, whereas significant increases were observed for Control berries between 0-21 dpg and 21-29 dpg (Figure 76a). However, a significant, three-fold, difference in proline concentrations between Girdle 1 and Control berries was only detected at the last sampling time point before harvest at 29 dpg (Figure 76a).

Only one out of the five proline-related genes, *VviPDH*, showed a difference in transcript accumulation between Girdle 1 and Control berries with significantly lower transcript numbers in Girdle 1 berries at the last two sampling time points (Figure 76b), suggesting little change to berry proline metabolism in response to girdling.

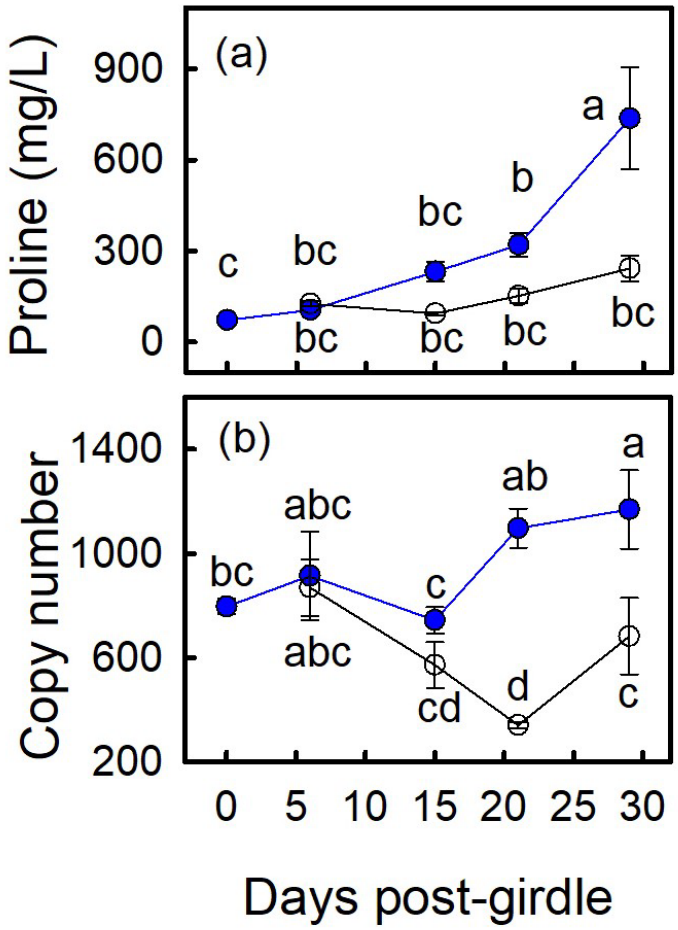


Figure 76. (a) proline concentration in the Control and Girdle 1 berry samples, b) expression of *VviPDH*, gene in deseeded Shiraz berries from Control (●) and peduncle-girdled (○) bunches (Girdle 1, 14th Jan). All data represent means ± SE (n = 3). Values are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post-hoc test.

Peduncle-girdling restricts proline import and affects berry GABA shunt

Proline and arginine are the main amino acids accumulating in ripening grape berries (Kliewer 1968, Stines *et al.,* 1999) and because of a linear correlation between proline concentration and the berry sugar to acid ratio, this amino acid is considered as a ripening indicator of grapes (Millery *et al.,* 1986). Significant changes in the expression of grapevine genes involved in proline biosynthesis, intercellular transport and degradation around the time of veraison, which coincided with an increase in berry proline concentrations (Figure 76a and data not shown), indicated that proline accumulation in grape berries was regulated by a developmental program rather than osmotic stress due to high berry sugar levels. Peduncle-girdling at early stages of the post-veraison phase (Girdle 1 and 2) changed the proline to arginine ratio from 1.3 to 0.2-0.5 (Table 38), which likely contributed to altered fermentation conditions and wine volatile production as described above. The most obvious explanation for the girdling-associated reduction in berry proline content was the disruption of the phloem. However, despite its common association with phloem transport, proline has also been detected in xylem sap of several plant species (Lehmann *et al.,* 2010) and from the developmental gene expression data presented in this study (Figure 76b) and previous work on proline regulation in grapes (Stines *et al.,* 1999), an effect on local proline

metabolism could not be excluded as the cause for the changes in berry proline levels. Since the expression of proline-related genes in tissue from the most severely proline-deprived Girdle 1 berries was largely unaffected by the girdling treatment (data not shown), restricted import was confirmed as the most likely reason for the observed changes in proline content. The reduced expression of *VviPDH* at 21 and 29 dpg was suggestive of a down-regulation of the proline degradation pathway, possibly regulated by the decreased proline concentration in the berries (Satoh *et al.,* 2002).

The role of proline in protecting plant cells from oxidative stress by activating enzymatic antioxidant systems or directly scavenging reactive oxygen species is well documented (Szabados and Savouré 2010, Signorelli *et al.,* 2015), hence it can be assumed that a reduction in proline levels, as was observed for Girdle 1 and 2 berries, would have compromised this protection. Indications for an increase in lipid oxidation and carotenoid degradation described support of this assumption. Oxidative stress conditions also provided a possible explanation for the decreased levels of two additional amino acids, glutamic acid and GABA, in Girdle berries (Table 38) as such stresses favour the activation of an enzymatic pathway known as GABA shunt (Bouché and Fromm 2004). In this pathway, GABA, produced from the decarboxylation of glutamic acid, enters the mitochondrial tricarboxylic acid cycle to generate succinate and NADH (Sweetlove *et al.,* 2010). Thereby, the pathway provides substrates (succinate and NADH) for mitochondrial respiration and ensures the ongoing production of energy for cell survival. The small increase in diethyl succinate concentration in Girdle 1 and 2 wines (Table 37) was conceivably caused by a metabolic shift to the GABA shunt resulting in elevated succinate levels in the berries.

Changes to ripening-related hormones in response to peduncle-girdling

Previous studies have suggested the involvement of ABA, auxins and cytokinins in the grape berry ripening process (Davies and Böttcher, 2014; Böttcher *et al.,* 2015a). In addition to their role in ripening control these hormones might also influence the production of wine volatile compounds through competition for common precursors for biosynthesis (Frébort *et al.,* 2011, Trinh *et al.,* 2012, Young *et al.,* 2012) or the regulation of secondary metabolism pathways (Bhargava *et al.,* 2013; Villalobos-Gonzalez *et al.,* 2016). Hence, the accumulation of ABA, auxins and cytokinins in response to peduncle-girdling was analysed in Girdle 1 and respective Control berries from the time of girdling until the last time point before harvest.

The accumulation of both active cytokinins, €P and *t*Z, was reduced in Girdle 1 berries to very different degrees (Figure 77a,b). The iP concentration remained low and unchanged over the 29 day period between girdling and the last sampling time point in Girdle 1 berries, but increased 10-fold in Control berries, followed by a 30% decrease at the last sampling time-point. In contrast, the concentration of *t*Z increased in Girdle 1 and Control berries between 15-29 dpg, but was 2-3-fold lower in Girdle 1 berries at the last two sampling time-points.

With the aim to evaluate the contribution of berry-localised changes in cytokinin metabolism to the observed differences in cytokinin levels, the expression of eighteen cytokinin-related genes was examined. Six showed a significant difference in abundance between Control and Girdle 1 berries at two or more time-points (Figure 77c-h). Among them, the phosphoribohydrolase gene *VviLOG5a* was the only gene associated with cytokinin metabolism and showing increased expression in Girdle 1 berries, whereas the remaining five genes related to cytokinin perception (*VviCHK2*) and signalling (*VviRR27*, *30*, *32* and *41*) and displayed a general reduction in transcript numbers in response to girdling.

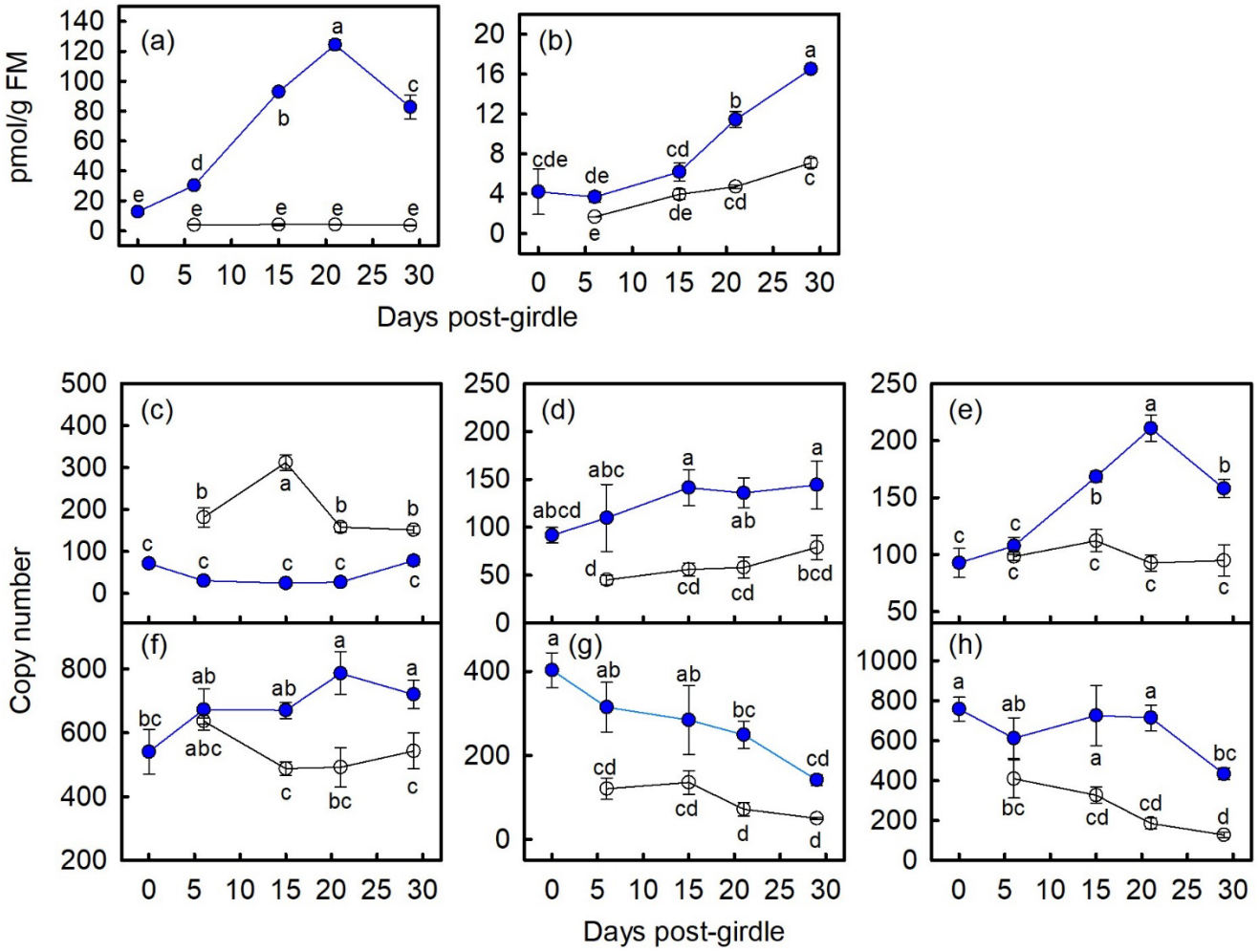


Figure 77. Concentration of (a) N^6-(△^{2}-isopentenyl)-adenine (iP) and (b) trans-zeatin (tZ) in deseeded Shiraz berries of peduncle-girdled (○) bunches [girdled 14 Jan (Girdle 1)], and of respective Control berries (●). Expression of cytokinin-related genes, (c) *VviLOG5a*, (d) *VviCHK2*, € *VviRR27*, (f) *VviRR30,* (g) *VviRR32* and (h) *VviRR41*, was analysed in the same berry samples and genes with a significant difference at two or more time-points are shown. Values represent means ± SE (*n* = 3) and are denoted by a different letter if the means differ significantly (P < 0.05) using one-way ANOVA followed by Duncan's post hoc test. FM, fresh weight.

The accumulation of the auxin IAA and its most prominent amino acid conjugate, IAA-Asp, in grape berries was largely unaffected by girdling. The only significant difference between Girdle 1 and Control berries occurred at 21 dpg, where there was a 1.6-fold reduction in the IAA-Asp concentration in Girdle 1 berries (Figure 78b). However, some differences in the expression of auxin conjugation (*VviGH3-1,* Böttcher *et al.,* 2010) and putative auxin biosynthesis (*VviYUC1*, *VviTAR1*, *VviTAR3,* Böttcher *et al.,* 2013c) genes were observed (Figure 78g-j). While transcript numbers of *VviGH3-1, VviYUC1* and *VviTAR3* were increased in Girdle 1 berries at two sampling time points, *VviTAR1* expression was decreased in response to girdling at the last three sampling time points.

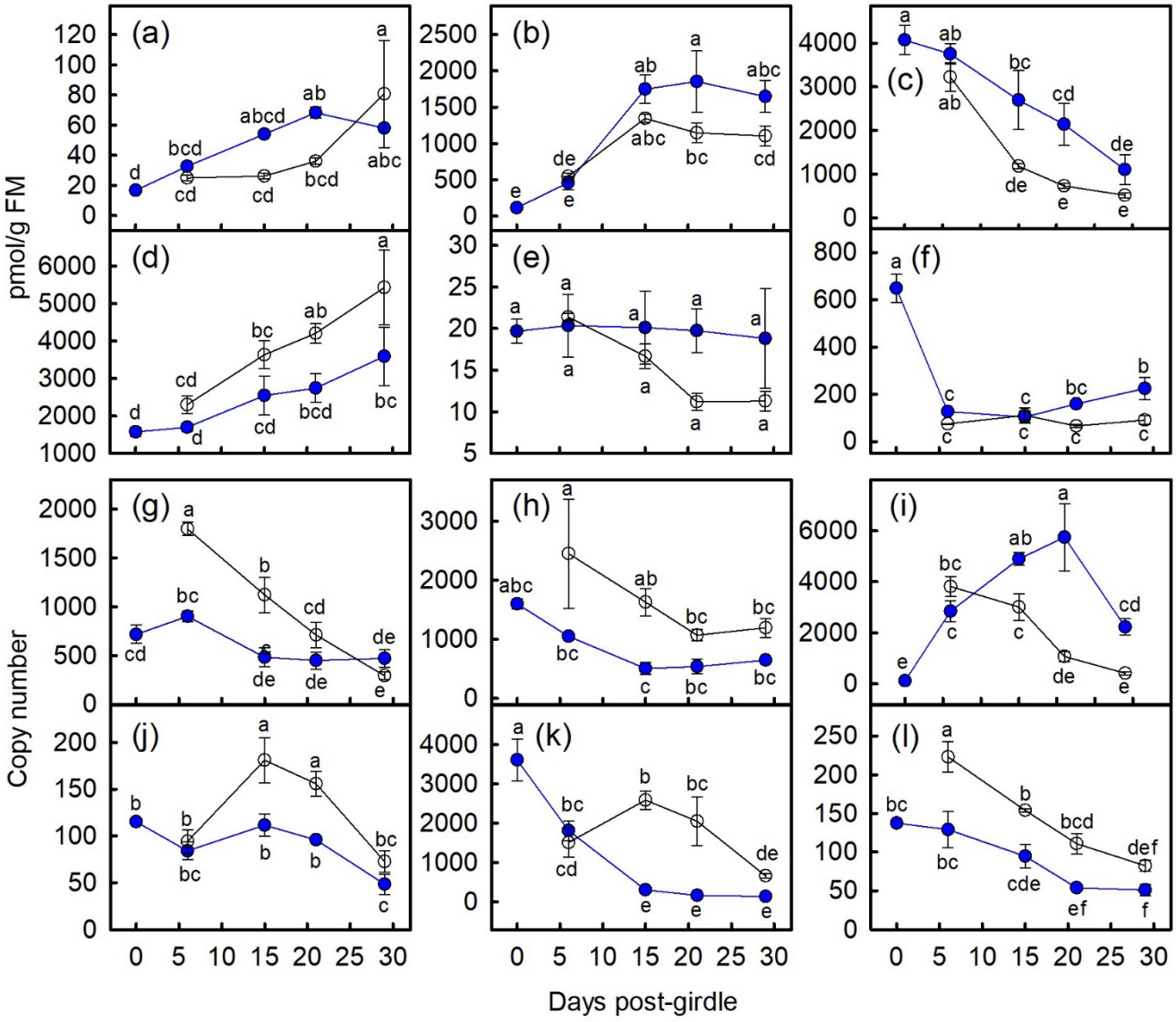


Figure 78. Concentration of (a) indole-3-acetic acid (IAA), (b) IAA-aspartic acid (IAA-Asp), (c) abscisic acid (ABA), (d) ABA-glucose ester (ABA-GE), (e) phaseic acid (PA) and (f) dihydro-phaseic acid (DPA) in deseeded Shiraz berries of peduncle-girdled (○) bunches [girdled 14 Jan (Girdle 1)], and of Control berries (●). Expression of (g) IAA-conjugation *VviGH3-1*, IAA biosynthesis ((h) *VviYUC1*, (i) *VviTAR1*, (j) *VviTAR3*)) and ABA biosynthesis-related genes ((k) *VviNCED1* and (l) *VviZEP*)) was analysed in the same berry samples. Values represent means ± SE (n = 3) and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test. FM, fresh weight.

The decrease in ABA concentration and the increase in the concentration of its glucose ester (ABA-GE) over time followed similar patterns in Girdle 1 and Control berries, but at two sampling time-points Girdle 1 berries showed decreased ABA levels compared with the Control, whereas ABA-GE accumulation was increased in Girdle 1 berries at the last sampling time-point (Figure 78c,d). The abundance of the ABA oxidation products phaseic acid (PA) and dihydro-phaseic acid (DPA) was largely not affected (Figure 78e,f).

Independent of girdling, the expression of the ABA biosynthesis genes 9-*cis*-epoxy-carotenoid dioxygenase, *VviNCED1*, and zeaxanthin epoxidase, *VviZEP*, was decreasing over time, but was higher in Girdle 1 berries than Control berries at two (*VviNCED1*) or three (*VviZEP*) sampling time-points (Figure 78k,l).

Peduncle-girdling has differential effects on ripening-associated hormones

Diminished phloem transport and a transformed cellular environment favouring oxidation were the most likely causes for the observed changes to the accumulation of ripening-related hormones in Girdle 1 berries described above.

The sugar-linked increase in ABA concentration in post-veraison grapes combined with the positive effect of ABA applications on various ripening parameters have established this hormone as an integral part of grape berry ripening (Davies and Böttcher 2014). Girdling-induced changes to the levels of ABA and its main metabolites were subtle (Figure 78c), in agreement with a previous study describing negligible effects of various modes of girdling on the distribution of ABA in grapevine (Düring *et al.,* 1978). A decrease in ABA content from the time of girdling was observed for Control and Girdle 1 berries, with relatively lower concentrations in Girdle 1 berries at two sampling time-points. This may be attributable to an increased glycosylation rate in response to girdling, as ABA-GE levels were higher in Girdle 1 than Control berries at 29 dpg (Figure 78d) or could have indicated that a proportion of berry ABA is normally imported from leaves via the phloem (Loveys and Düring 1984). However, since ABA is derived from the cleavage of carotenoids (Xiong and Zhu 2003), and in agreement with the increased C13-norisoprenoid levels detected in Girdle wines, a metabolic shift in the breakdown of carotenoids was another possible cause for the ABA reduction in Girdle 1 berries. While substrate specificities and enzyme activities of C13-norisoprenoid-generating carotenoid cleavage dioxygenases (CCDs) in grapes are yet to be fully understood (Yuan and Qian 2016), a grapevine CCD (VviCCD1) catalysing the cleavage of zeaxanthin, the carotenoid substrate for ABA production (Marin *et al.,* 1996), to produce a hydroxylated form of β–ionone has been reported (Mathieu *et al.,* 2005). It is possible therefore, that a relative increase in CCD activities compared with ZEP and NCED1 activities in Girdle 1 berries resulted in the increased concentration of β–ionone in Girdle 1 wines and the decreased concentration of ABA in Girdle 1 berries. In this context, the elevated expression of VviZEP and VviNCED1 in Girdle 1 berries (Figure 78k,l) could be interpreted as a counteracting response to the decreased biosynthesis of ABA but could also have reflected the dehydrated state of Girdle 1 berries (Behnam *et al.,* 2013).

Many studies investigating the role of auxins in grape berry ripening have identified IAA as a ripening inhibitor with low concentrations in ripening grapes (Davies and Böttcher 2014). On the other hand, the accumulation of the IAA amino acid conjugate IAA-Asp has been found to coincide with sugar uptake in post-veraison berries and might represent a mechanism for the removal of active auxin, thereby abolishing the auxin-imposed ripening inhibition (Böttcher *et al.,* 2010). Low IAA concentrations in post-veraison berries and a significant increase in berry IAA-Asp content were also detected in this study, irrespective of the girdling treatment (Figure 78a,b). The only significant difference between Control and Girdle berries was a slight reduction in the IAA-Asp concentration at 21 dpg in Girdle 1 berries. The essentially unchanged accumulation of auxins in Girdle 1 berries compared with Control berries indicated an autonomous regulation of auxin levels, and hence auxin-dependent ripening control, in grapes. It was also in contradiction to the increased expression of auxin conjugation (VviGH3-1) and biosynthesis (VviYUC1, VviTAR3) genes in response to the girdling treatment (Figure 78g-j). A possible explanation for this discrepancy is the susceptibility of IAA and IAA-Asp to oxidation (Östin *et al.,* 1998; Pěnčík *et al.,* 2013), which would mask a potentially increased production of these compounds in Girdle 1 berries.

Cytokinins, in particular iP, were the ripening-related hormones most impacted by post-veraison peduncle-girdling. The physiological function of the post-veraison accumulation of iP reported previously (Böttcher *et al.,* 2013a, 2015a) and also observed in this study (Figure 77a) is unknown, but might be related to the expansion-driven growth of ripening grapes and the establishment of berries as strong sink organs (Böttcher *et al.,* 2015a). Girdling completely halted the accumulation of iP in berries (Figure 77a), presumably the main source for berry iP is via phloem transport. In accordance with this, iP ribosides and ribotides have been detected in the phloem of *Arabidopsis* (Hirose *et al.,* 2008). Conversely, a small increase in the concentration of tZ was found over time in Girdle 1 and Control berries (Figure 77a). The reduced tZ levels in Girdle 1 berries, compared with Control berries, at 21 and 29 dpg and the steady state iP concentration in shrivelling Girdle 1 berries were suggestive of girdling-induced changes to cytokinin metabolism. Enzymatic reactions may not be involved since genes related to cytokinin biosynthesis, degradation and iP to tZ conversion (Böttcher *et al.,* 2015a) were either unresponsive to girdling or were not expressed at detectable levels in the tested berry tissue. The only exception was the cytokinin activation gene VviLOG5a, which was linked to the post-veraison increase in iP concentration in a previous study (Figure 77c; Böttcher *et al.,* 2015a). The increased expression of this gene in Girdle 1 berries at all time points after girdling suggested induction by low iP levels. The opposite was observed for the expression of a cytokinin receptor gene and four response regulator genes, which was repressed by girdling and confirmed the reduced cytokinin content in Girdle 1 berries compared with Control berries (Figure 77d-h). The net reduction in berry cytokinin content after peduncle-girdling was thus possibly due to the direct oxidation of tZ and iP by reactive oxygen species as has previously been described to occur during the imbibition of soybean (Glycine max (L.) Merr.) seeds (Gidrol *et al.,* 1994).

Wine volatiles with ubiquitous response to berry sugar manipulation

Interestingly, 25 out of the 58 volatile compounds (43%) found to be significantly changed in wines made from sugar-uptake-restricted Girdle berries had previously been identified as significantly different in wines made from Shiraz or Riesling berries, where sugar uptake was delayed by NAA treatment (Table 37).

Most of these compounds (21) had increased concentrations in Girdle wines and no clear association with NAA-induced ripening delay since seven compounds were more abundant in NAA wines, 11 compounds were less abundant in NAA wines and three compounds had a mixed response to NAA treatment across the three different studies (Table 37; Böttcher *et al.,* 2010, 2012; Davies *et al.,* 2015).

However, the comparison revealed eight volatiles with changed abundance due to reduced sugar uptake that was independent of the method used to achieve the sugar reduction. This core group of eight compounds might therefore represent the effect on wine volatiles of a universal response to delayed or restricted sugar accumulation in grape berries. Ethyl acetate, 2-methylpropyl acetate, 3-methylbutyl acetate, ethyl butanoate, 1-propanol, benzyl alcohol and β-damascenone were found at higher concentrations in wines made from berries with NAA-delayed or girdling-restricted sugar accumulation, whereas ethyl 2-furoate was the only compound consistently reduced in abundance in those wines (Table 37). Three out of the seven compounds with increased abundance in NAA and Girdle wines, 2-methylpropyl acetate, 3-methylbutyl acetate and 1-propanol, have previously been identified as members of a group of wine volatiles characterised by high relative concentrations early in the ripening phase and by a negative correlation with berry sugar levels at later ripening stages (Boss *et al.,* 2014). In agreement with its reduced concentration in NAA and Girdle wines, ethyl 2-furoate has been described as positively correlated with berry sugar levels (Boss *et al.,* 2014).

A possible explanation for the large differences in the effect on volatile aroma compounds between NAA-induced and girdling-induced delays in sugar accumulation is that NAA treatments work predominantly by extending the pre-veraison period (Böttcher *et al.,* 2010, 2012a; Davies *et al.,* 2015), rather than by stopping or slowing sugar uptake during the post-veraison period.

Some interesting observations can be made when comparing the wine volatile compound profiles reported in the current study with those in the study of Boss *et al.* (2018), where wines were made from grapes taken at different stages during berry development. In that study, volatile compounds were grouped into six clusters based on the changes observed in their concentrations during development, from two weeks post flowering through to commercial harvest, in Cabernet Sauvignon and Riesling wines. If girdling is pausing berry development at the stage when girdling occurs, changes in volatile abundances associated with later developmental stages should not be observed in wines made from Girdle berries.

Cabernet Sauvignon (CS) Cluster A was the only cluster where compounds were increasing in concentration during the equivalent period of development the girdling experiment took place. Eight of the 15 compounds that were higher in the Control wines than in one or more of the Girdle wines were grouped in CS Cluster A (Table 39; Boss *et al.,* 2018). Thirty three of the 43 compounds that were significantly higher in one or more of the Girdle wines compared to the Control wines were found in CS Clusters B-F where, in general, the compounds decreased in concentration during this period of fruit development (Table 39; Boss *et al.,* 2018). The similarities between the two studies regarding the developmental trends of wine volatile compounds suggest that girdling is inhibiting ripening-related changes in fruit composition that can influence wine volatile composition. It is possible that the compounds which remained higher in the Girdle wines did so because the berries did not increase in size and hence there was no dilution effect. This could also account for compounds that were higher in the Control wines, if an inhibitor of their production was not being diluted via berry expansion in the Girdle samples. However, two of the compounds with increased abundance in Control wines, 3-(methylthio)-1-propanol and ethyl 3-(methylthio)propanoate, are derived from methionine. The concentration of this amino acid was decreased in the Girdle 1 and 2 berry samples compared with the Control (Table 38), which is suggestive of a direct effect of girdling on the import of methionine into the berries.

Table 39. Volatile compounds found to be significantly different ($p < 0.05$) in the headspace of the wines (n=18).

Compound	Quantifier ion[A]	LRI[B]	ID[C]	CS Cluster (from Boss *et al.*, (2018))
Ethyl acetate	61	887	A	C
Ethyl propanoate	102	957	A	A
Ethyl 2-methylpropanoate	71	966	A	D
Propyl acetate	61	973	A	C
2,4,5-Trimethyl-1,3-dioxolane	101	1009	B	E
2-Methylpropyl acetate	73	1012	A	D
2,4,6-Trimethyl-1,3-dioxolane	101	1026	B	-
Ethyl butanoate	88	1036	A	C
1-Propanol	59	1043	A	E
Butyl acetate	56	1075	A	C
3-Methylbutyl acetate	87	1121	A	C
Ocimene	121	1135	A	C(R)[D]
2-Isobutyl-4,5-dimethyl-1,3-dioxolane	101	1143	B	-
3-Methylbutanol[E]	70	1198	A	A
Hexyl acetate	84	1256	A	B
Ethyl 3-hexenoate	88	1289	A	E
(*E*)-3-Hexenyl acetate	67	1290	A	B
(*Z*)-3-Hexenyl acetate	67	1298	A	E
Propyl hexanoate	117	1303	A	C
(*E*)-2-Heptenal	83	1304	A	C
Ethyl heptanoate	113	1317	A	F
Ethyl lactate	75	1324	A	E
Ethyl 2-hexenoate	97	1327	A	A
1-Hexanol	69	1333	A	F
3-Ethoxy-1-propanol	59	1354	A	E
Heptyl acetate	98	1257	A	F
(*Z*)-3-Hexenol	82	1363	A	E
1-Octen-3-ol	72	1430	A	C
1-Heptanol	83	1434	A	E
Decanal	82	1480	A	A
1-Butyl cyclohexene	138	1482	C	-
Benzaldehyde	106	1503	A	B
Propyl octanoate	127	1504	A	E
Vitispirane I and II	192	1507	B	D
Linalool	121	1528	A	F
Propanoic acid	74	1535	A	-
1-Octanol	84	1537	A	F
Ethyl 3-(methylthio)propanoate	148	1547	A	A
2-Undecanone	71	1581	A	D
β-Cyclocitral	152	1598	B	F
Ethyl 2-furoate	95	1602	A	F
1-Nonanol	70	1639	A	E
Diethyl succinate	129	1656	A	D
2-Methylbutanoic acid	74	1660	A	A(R)
Ethyl 9-decenoate	88	1672	B	-
3-(Methylthio)-1-Propanol	106	1693	A	A
Propyl decanoate	173	1706	B	-
Geranyl acetate	93	1734	A	F
ß-Citronellol	123	1742	A	F
Ethyl phenyl acetate	91	1761	A	A
β-Damascenone	121	1801	A	A
Benzyl alcohol	107	1847	A	F

Compound	Quantifier ion[A]	LRI[B]	ID[C]	CS Cluster (from Boss *et al.*, (2018))
2-Phenylethyl propanoate	104	1855	B	A
β-Ionone	177	1905	A	F
2-Phenylethyl butanoate	104	1929	A	A
Octanoic acid	101	2052	A	A
2-Phenylethyl hexanoate	104	2155	A	A
2-Phenylethyl octanoate	104	2375	A	A

[A]Unique ion (m/z): used for peak area determination. [B]Linear Retention Index (LRI) calculated from retention relative to the retention of a series of n-alkanes (C_8-C_{26}). [C]A: Identity confirmed by matching weight spectra and LRI with that of authentic standards; B: Tentative assignment based upon comparison with NIST05 and Wiley Registry (7th edition) weight spectral libraries, and published retention times; C: Tentative identification of compound class suggested by comparison of weight spectra with those of related compounds in compound libraries. [D]If the compound was not present in the Cabernet Sauvignon (CS) Clusters, the Riesling cluster has been cited and indicated by (R). A dash (-) means this compound was not in a cluster from either variety. [E]Compounds in italics are significantly higher ($p < 0.05$) in the Control than one or more of the girdled samples.

6.13.4 Conclusions

This study has demonstrated that the sugar content of Shiraz berries determined the quantitative and qualitative effects of peduncle-girdling on wine volatile compounds. Preventing further sugar accumulation at 10-14 °Brix was shown to have severe effects on the accumulation of ripening-related grape metabolites such as amino acids and hormones and, by inference, the cellular redox status. Consequently, wine made from these berries displayed alterations to a large number of compounds and compound classes, in particular esters, higher alcohols and C13-norisoprenoids, that would potentially result in substantial changes to wine flavour and aroma. However, when grapes reached 18 °Brix, peduncle-girdling led to only minor modifications of berry amino acid content and wine volatile composition. By comparing wines made from berries of peduncle-girdled bunches with those made from berries where ripening was delayed by auxin treatments, a small group of volatile compounds was identified that universally responds to treatments delaying sugar accumulation in grapes. The findings suggest that once berries have reached a modest sugar content, preventing further sugar uptake even by an invasive treatment, such as girdling, has minimal impact on wine volatile composition. This study confirms that the manipulation of sugar accumulation can mitigate the climate change-related acceleration of 'sugar-ripeness' in warm to hot wine regions while maintaining wine volatile composition.

6.14 Post-veraison restriction of phloem import into Riesling (*Vitis vinifera* L.) berries induces transient and stable changes to fermentation-derived and varietal wine volatiles

6.14.1 Introduction

The previous section detailed an experiment in Shiraz where post-veraison peduncle girdling was used to prevent sugar accumulation at certain times during ripening. This was seen as a useful tool to study to better understand and manage the relationship between grape berry 'sugar-ripeness' and 'flavour-ripeness'. The findings suggested that the main impact of sugar uptake on wine volatile formation might be restricted to the phase of rapid sugar accumulation The ultimate aim is to regulate the accumulation of grape compounds critical for the production of key wine aroma and flavour compounds under changing climatic conditions without having excessive alcohol content.

The experiment described below also made use of girdling as a tool to further study the relationship between berry sugar concentration and wine aroma/flavour compounds and the influence of other key grape metabolites in Riesling. This allowed the determination of critical times during the ripening phase with regard to wine volatile production and the identification of potential targets for the manipulation of varietal Riesling aroma compounds.

6.14.2 Materials and methods

Grapes and girdling

Bunches of *Vitis vinifera* L. cv. Riesling vines grown on own roots at Gumeracha, South Australia were girdled on 4 February 2013 by removing a 1–2 mm wide section of the phloem from the peduncle circumference with a razor blade. Girdling was performed in three adjacent rows, with 14 bunches from 5–6 adjacent vines constituting one replicate. On the same vines, the same number of untreated bunches at a similar developmental stage to the girdled bunches was used as Controls.

Berries (four per bunch, 56 per replicate) from Girdle and Control bunches were sampled at 0 (Control only), 10, 20 and 30 days after girdling, weighed, frozen in liquid N_2 and stored at -80°C.

Liquid chromatography (LC)-electrospray ionisation (ESI)-MS/MS analysis of cytokinins

Powdered berry tissue (100 mg) was extracted and N^6-(Δ^2-isopentenyl)-adenine (iP) quantified by LC-ESI-MS/MS as described by Böttcher *et al.* (2015a).

Fermentation conditions and headspace volatile analysis

Subsamples (25 g) of the ground Riesling berries were fermented and analysed for headspace volatiles by SPME. All samples were adjusted to a yeast-available nitrogen (YAN) concentration of 300 mg N/L using ammonium chloride and a combined glucose/fructose concentration of 190 g/L.

6.14.3 Results and discussion

Phloem-restriction of Shiraz berries at different post-veraison stages has provided new insights into the dependency of wine volatile formation on sugars and other phloem-transported compounds (this report). In order to better define berry developmental stages associated with phloem-influx dependent wine volatile formation, two Riesling berry samples, one non-girdled (Control) and one girdled so that the berries had a stable sugar content but restricted access to phloem sugar and other compounds, were analysed for selected grape and wine compounds at several time-points during the ripening phase. The

peduncle-girdling of Riesling bunches occurred when the berries had reached an average TSS level of 13.6 °Brix (4 Feb) and Control and Girdle berries were sampled and fermented at 0 (Control only), 10, 20 and 30 days post-girdling (dpg).

Differential response of grape parameters to peduncle-girdling

The weight and sugar concentration of Control berries increased significantly between 0–20 dpg, whereas Girdle berries displayed a small, but significant, decrease in weight and TSS during the first ten days after girdling and only minor fluctuations in both parameters thereafter, resulting in significantly lower weight and TSS values than those of Control berries at every sampling time-point (Table 40). Unlike the observed arrest in berry growth and sugar accumulation, YAN concentration halved in girdled Riesling berries over the course of the experiment, whereas the YAN decrease in Control berries was comparatively small (Table 40). This result was different from girdled Shiraz berries of similar sugar content, where YAN concentration was not affected by girdling, presumably due to severe shrivelling of the girdled Shiraz berries (this report).

Table 40. Grape and wine composition of Control and Girdle Riesling berries/wines.

Time point (dpg)	Treatment	Grape parameters					Wine parameters	
		Weight (g/berry)	TSS (°Brix)	YAN (mg N/L)	Gluc+Fruc (g/L)	iP (pmol/g FM)	Ethanol (% v/v)	pH
0	Control	1.25c	13.63d	228.01a	98.23c	2.60c	11.00c	3.05bc
10	Control	1.39b	16.20c	198.90bc	132.88b	2.84c	11.27bc	3.10b
	Girdle	1.17d	12.13e	170.14d	82.84cd	0.23e	11.77abc	3.00c
20	Control	1.54a	19.33b	209.64b	168.39a	6.90b	12.03ab	3.33a
	Girdle	1.11e	11.90e	127.40e	88.30cd	0.27e	11.87ab	2.99c
30	Control	1.56a	20.23a	190.36c	178.46a	14.53a	12.30a	3.37a
	Girdle	1.17d	12.13e	114.59e	78.19d	0.91d	12.10a	3.00c

Values are the mean of n = 3 biological replicates. Values with different letters are significantly different ($P < 0.05$). dpg, days post-girdle; FM, fresh weight; Fruc, fructose; Gluc, glucose; iP, N^6-(Δ^2-isopentenyl)-adenine; YAN, yeast-available nitrogen.

Ammonia contribution to YAN decreased over time from 115 to <40 mg/L in Control and Girdle fruit alike (data not shown), which indicated that the significant reduction in the concentration of 18 berry amino acids was the main reason for the lower YAN concentration in Girdle berries when compared with that of Control berries (Table 41). The reduced accumulation of amino acids was an early effect of the girdling treatment, with all but glutamic acid and histidine detected at significantly lower concentration in fruit from girdled bunches compared to Control fruit from 10 dpg onwards. This was different to the girdling response in Shiraz berries, where out of the eight amino acids with altered concentrations, four were increased in Girdle berries (this report). As discussed above for YAN, the different accumulation patterns of amino acids in girdled Shiraz and Riesling grapes might be explained by the lack of shrivelling in Riesling berries. Severe shrivelling, as observed in Shiraz berries from girdled bunches, affects the concentration of stored amino acids within the fruit, but could also trigger further accumulation of amino acids through protein breakdown and disturbance of metabolic pathways involving amino acids. It should be noted that Riesling and Shiraz berries differ significantly in their amino acid profiles and overall amino acid concentration, Shiraz berries have a significantly higher concentration than that in Riesling (Spayd *et al.,* 1994; Stines *et al.,* 2000; Antalick *et al.,* 2015; this report). However, in both cultivars proline was the amino acid with the largest decrease in response to girdling, with an up to 61-fold difference between

Riesling Control and Girdle berries, possibly rendering Girdle berries more susceptible to oxidative stress (Signorelli *et al.,* 2015; this report).

Table 41. Amino acids with significantly higher concentration at one or more time-points in the juice of Riesling berries from bunches that were peduncle-girdled (Girdle) compared to that of Control berries.

Amino acid	Concentration (mg/L)						
	0 dpg	10 dpg		20 dpg		30 dpg	
	Control	Control	Girdle	Control	Girdle	Control	Girdle
Alanine	50.6c	72.4b	31.7d	114.6a	15.3e	114.8a	26.1de
β-Alanine	0.4d	1.5b	1.0c	3.1a	1.2bc	2.8a	2.3b
γ-Aminobutyric acid	89.7b	99.4ab	40.0c	113.0a	36.5c	107.0a	34.0c
Arginine	224.9bc	267.2b	172.9cd	349.6a	108.2d	363.5a	112.2d
Aspartic acid	55.0b	54.9b	28.2d	60.3a	29.1d	49.5c	26.3d
Glutamic acid	45.4c	76.7b	66.1b	110.6a	40.5c	109.6a	47.8c
Glutamine	150.2a	94.4b	32.1d	90.9b	22.6d	72.2c	18.9d
Histidine	36.8bc	42.0ab	32.2bc	48.6a	28.3c	50.6a	26.6c
Hydroxyproline	2.0ef	2.4c	2.1de	2.8b	1.8f	3.1a	2.2cd
Isoleucine	4.3c	8.6b	5.4c	11a	3.9c	12.3a	4.2c
Leucine	6.7d	9.3c	5.6d	14.2b	4.6d	17.0a	5.2d
Methionine	0.4d	0.9c	0.4d	1.3b	0.6cd	2.2a	0.6cd
Phenylalanine	4.6c	9.5b	4.8c	14.5a	3.5c	15.7a	4.7c
Proline†	73.0c	92.2c	20.1d	157.0b	8.8d	201.1a	3.3d
Serine	33.7c	42.2b	16.2d	53.3a	8.4e	53.7a	10.1e
Threonine	34.9c	50.5b	24.1d	61.0a	10.1e	59.2a	9.9e
Tyrosine	5.1c	7.6b	4.8cd	14.3a	3.2d	14.8a	3.6cd
Valine	7.5c	12.7b	7.5c	22.1a	5.5c	24.4a	6.8c

Values are the mean of n = 3 biological replicates. Values with different letters are significantly different ($P < 0.05$). † quantified using ninhydrin method as specified in Materials and methods section. dpg, days post-girdle.

Time-dependent effect of peduncle-girdling on wine volatiles

Model wine batches (50 mL), adjusted for their glucose/fructose and YAN concentration, were produced from Control and Girdle berries at 0 (Control only), 10, 20 and 30 dpg. All fermentations proceeded to dryness and while there was an increasing trend for ethanol concentration over the 30 days of the study, no significant difference between Control and Girdle wines was observed at any time-point (Table 40). Despite identical hang times of Control and Girdle berries, wine pH was consistently lower in Girdle wines compared with Control wines. This might be explained by the lower YAN concentration of Girdle musts (Table 40) and hence increased proportion of ammonium nitrogen in YAN-adjusted Girdle ferments compared with Control ferments. High ammonium to α-amino acid nitrogen ratios have been associated with elevated proton ion extrusion by yeast cells during fermentation (Peña *et al.,* 1987; Torija *et al.,* 2003). It could also indicate that girdling had an effect on berry acid balance, possibly due to the restricted import of potassium and hence reduced potassium bitartrate formation (Rogiers *et al.,* 2017).

The wines were analysed for their volatile compounds using SPME GC/MS and 72 of 166 identified compounds were significantly different ($P < 0.05$) in concentration in the headspace of the wines made from Control fruit compared with wines made from Girdle fruit (Table 42). Of these compounds, 35 were more abundant in one or more of the Girdle wines, whereas 37 compounds showed higher abundance in Control wines. In the case of the four compounds with the smallest, 1.3–1.4-fold, increase in abundance in Girdle wines (3-ethoxy-1-propanol, 1-heptanol, *β*-damascenone, and nerolidol), their elevated concentration may have been the result of a dilution effect due to the expansion of Control berries, which

had a 1.2–1.4-fold higher berry weight (Table 40). Although dilution in the expanding Control berries might also have contributed to the increased concentration of the remaining 31 compounds with higher abundance in Girdle wines, additional changes to Girdle berry composition must have occurred to explain the 1.5–6.1-fold concentration differentials between Control and Girdle wines (Table 42). While the effect of girdling on most of the analysed grape parameters (Tables 40, 41) was observed at the first sampling time-point after girdling (10 dpg), differences in wine volatiles became more pronounced from 20 dpg onwards (Table 42). Forty-six out of 72 compounds (64%) had a significant concentration difference between Control and Girdle wines at either or both, 20 dpg and 30 dpg, with 24 compounds more abundant in Girdle wines and 22 compounds more abundant in Control wines. Among the 24 compounds with increased abundance in Girdle wines, monoterpenes and their derivatives were most impacted by the girdling treatment with 11 representatives (*α*-terpinene, limonene, *γ*-terpinene, *β*-ocimene, hotrienol, linalool, 4-terpineol, geranic oxide, terpinolene, *α*-terpineol and (*E*)-geraniol) (Table 42). Three additional monoterpenoids (*m*-cymene, geranyl ethyl ether and nerol oxide) were detected at higher concentration in Girdle wines at all three time-points compared with the Control wines, and nerolidol, a sesquiterpene, was also more abundant in Girdle wines (Table 42). The induction of terpenoid accumulation by girdling has also been reported in Shiraz (this report) and has several possible explanations. It is feasible that phloem-restriction of Riesling berries at 13.63 °Brix prevented or slowed the developmentally controlled decrease of monoterpenoids and other compounds with increased abundance in Girdle wines. The significant reduction in proline and iP concentration in girdled berries, with the differential between Girdle and Control berries more than doubling between 10 and 20 dpg, infers a weakened antioxidant system in these grapes (Gidrol *et al.,* 1994; Szabados and Savouré 2010; Signorelli *et al.,* 2015; this report) and oxidative stress is a possible cause for the observed increase in terpene concentration. In particular, monoterpenes have been associated with the antioxidant defence system as their concentration in leaves of *Houttuynia cordata* Thunb. (HCT) and Amazonian trees has been found to increase in response to salicylic acid- (Xu *et al.,* 2012) or temperature-induced (Jardine *et al.,* 2017) oxidative stress, respectively. Moreover, the inhibition of monoterpene biosynthesis in *Hevea brasiliensis* Müll. Arg. leaves resulted in increased concentrations of reactive oxygen species (Chen *et al.,* 2009).

Table 42. Volatile compounds found to be significantly different in the headspace of wines produced from Riesling berries of bunches that were peduncle-girdled (Girdle) and from Control berries.

Compound	Area under the peak of the selected ion relative to the relevant internal standard						
	0 dpg	10 dpg		20 dpg		30 dpg	
	Control	Control	Girdle	Control	Girdle	Control	Girdle
Higher in Girdle wines than Control wines at one or more time points							
Alcohols/aldehydes							
3-Ethoxy-1-propanol†	31.4cd	34.7bc	36.8b	30.5cd	42.4a	28.0d	38.0b
1-Heptanol§	8.0b	6.8b	11.7a	7.0b	10.1a	6.6b	8.3b
(*E*)-2-Heptenal†	199.0b	174.1b	178.9b	161.4b	203.2b	176.3b	305.7a
(*E*)-2-Octenal	67.1ab	55.8ab	69.0ab	45.9b	71.1ab	48.5b	86.1a
Esters							
Ethyl 9-decenoate§	78.4c	82.9c	174.3a	102.8bc	161.9a	105.9bc	135.6ab
Ethyl 2-hydroxy-4-methylpentanoate§	13.0ab	8.2c	11.5bc	8.7bc	17.1a	9.0bc	16.8a
Ethyl lactate†	114.9ab	67.9cd	97.2bc	54.7d	134.3a	48.2d	134.7a
Ethyl 3-methylbutanoate§	7.3a	4.4b	6.0ab	4.1b	6.5ab	4.2b	7.5a
Ethyl 2-methylpropanoate†	13.3a	8.8bc	11.7abc	8.4c	12.6ab	8.0c	14.9a
Methyl hexanoate§	20.7b	21.2b	23.1ab	22.6ab	25.3ab	20.1b	32.4a
Monoterpenoids							
m-Cymene	45.2de	38.0e	68.2c	44.3de	93.3b	54.4d	116.3a
Hotrienol	1886.4d	2666.0cd	3448.2cd	3242.6cd	5212.3b	4041.1bc	7140.4a
Geranic oxide	1.6c	1.3c	2.5c	2.0c	5.8b	3.8bc	12.4a
(*E*)-Geraniol	43.9e	56.6de	58.2de	96.1cd	117.1bc	158.4b	221.6a
Geranyl ethyl ether	382.4d	481.2d	785.7c	760.4c	1188.7b	857.7c	1598.1a
Limonene§	505.6c	394.3c	696.8c	629.1c	1707.9b	1017.9bc	3536.5a
Linalool†	1955.6c	2513.7c	4675.2c	5564.9c	11824.4b	11464.6b	23924.9a
3-Methylene-1,5,5-trimethylcyclohexene§	98.4c	84.5c	113.1c	122.6bc	251.2a	117.3bc	180.7b
Nerol oxide	24.8d	33.7d	49.7bc	39.5cd	59.8b	52.3bc	79.1a
β-Ocimene†	84.9d	117.6d	265.9cd	312.0cd	803.2b	704.6bc	2108.5a
α-Terpinene	222.8c	156.2c	247.5c	216.8c	554.3b	329.6bc	1217.3a
γ-Terpinene	258.6c	260.5c	619.0bc	257.4c	980.8b	326.8c	1824.5a
α-Terpineol§	345.7c	382.3c	940.4c	779.3c	2651.1b	1773.9bc	6411.9a
4-Terpineol	170.0cd	140.1cd	225.2bc	106.6d	294.0b	120.2d	409.1a
Terpinolene	353.1bc	296.3c	504.9bc	310.6bc	1008.1b	630.4bc	3129.5a
Nor-isoprenoids							
β-Damascenone†	37.6e	64.7de	72.4d	104.3c	120.5bc	134.7b	173.9a
1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)	2.0d	2.8cd	5.5c	3.9cd	10.6b	4.8cd	17.9a
Vitispirane†	15.9d	18.4d	66.2c	23.8cd	139.3b	42.1cd	255.0a
Aromatics							
Methyl salicylate§	2.1ab	1.7ab	1.5ab	1.5ab	2.2a	1.4b	2.2a
1-Phenylethanone	46.6b	34.9c	40.9bc	37.0c	54.5a	36.9c	42.6bc
Others							
Acetic acid§	63.8b	68.7b	95.6ab	87.5ab	98.1ab	76.8b	117.6a
γ-Butyrolactone§	17.9a	12.6c	16.1ab	13.0bc	14.4bc	14.1bc	12.2c
1,1-Diethoxy-2-ethylhexane§	125.4b	86.0b	283.8a	110.3b	99.9b	141.0b	160.8b
1-Ethoxy-1-pentoxyethane	9.5ab	5.0b	12.4a	4.8b	3.9b	6.9ab	7.2ab
Nerolidol§	9.1ab	9.1ab	10.3a	6.4cd	7.5bcd	6.1d	7.7bc
Higher in Control wines than Girdle wines at one or more time points							
Alcohols							
1-Butanol§	58.6ab	49.1b	52.4b	64.2a	53.6b	64.2a	54.6ab
1-Hexanol†	1815.7b	2041.8a	1939.5ab	2136.0a	1450.8c	1964.8ab	1461.9c
Methionol§	17.2f	28.9d	21.3e	41.2b	33.1c	46.3a	31.8cd
3-Methyl-1-butanol	1589.4a	1496.6ab	1444.2ab	1551.2a	1355.2b	1464.3ab	1426.6ab
3-Methylbutyl hexanoate§	59.4a	56.0a	43.0ab	53.5a	33.5b	48.0b	35.7b
3-Methyl-pentanol§	143.1c	153.1bc	118.6d	174.0ab	133.9cd	178.1a	166.2ab
4-Methyl-pentanol§	73.8bc	74.8bc	61.4d	87.5a	72.6c	85.5ab	84.9abc
2-Methyl-1-propanol§	203.6a	159.8b	112.9c	99.7c	110.8c	98.0c	105.4c
1-Nonanol†	81.3f	120.1c	96.6e	157.7a	110.4cd	134.4b	100.0de
1-Octen-3-ol†	6.9d	9.6c	10.9c	13.3b	10.6c	15.7a	11.6bc
1-Pentanol§	5.6c	7.3b	6.3c	9.0a	5.4c	8.5a	6.0c
1-Penten-3-ol	11.9a	11.0ab	7.3bc	9.5ab	5.5c	8.9ab	8.2abc
Esters							
Ethyl 2-furoate‡	10.0a	8.4ab	4.5c	10.7a	5.8bc	10.6a	7.1bc
Ethyl (*Z*)-3-hexenoate†	4.2a	3.1bc	2.6c	3.4ab	2.0c	3.1bc	2.2c
Ethyl 3-(methylthio)propanoate‡	0.5ab	0.7a	0.6b	0.9a	0.9a	1.0a	0.9a
Ethyl nonanoate§	51.9b	62.6ab	44.5b	77.5a	54.7b	63.1ab	51.6b

Compound	Area under the peak of the selected ion relative to the relevant internal standard						
	0 dpg	10 dpg		20 dpg		30 dpg	
	Control	Control	Girdle	Control	Girdle	Control	Girdle
(*E*)-3-Heptenyl acetate	69.6a	52.7b	28.2c	29.3c	19.8c	21.6c	20.4c
(*Z*)-3-Hexenyl acetate†	67.5d	157.8a	132.6ab	150.8a	86.5cd	109.1bc	88.8cd
Hexyl acetate†	6631.9ab	7142.7a	5652.2ab	6617.0ab	3538.2c	5290.4bc	3665.4c
Hexyl hexanoate§	13.4ab	15.0a	11.4abc	10.6bc	5.9d	9.1cd	6.0d
Methyl decanoate	11.7bc	13.9abc	10.9c	18.0a	11.6bc	15.3ab	12.3bc
Pentyl acetate§	13.5a	14.7a	8.4b	14.7a	5.9b	12.2a	6.9b
Monoterpenoids							
Citronellyl acetate	10.2ab	11.7a	8.5ab	11.5a	7.2b	10.3ab	7.6b
Geranyl acetate‡	9.1ab	9.6a	6.7bc	9.9a	5.2c	8.0ab	5.2c
(*E*)-Pyran linalool oxide	3.3b	4.5a	2.5c	2.9bc	1.3d	2.3c	0.7d
(*Z*)-Pyran linalool oxide	5.5a	3.9b	2.0c	1.7c	0.4e	1.2d	0.2e
Aromatics							
Benzyl alcohol†	4.1b	4.2b	3.7b	7.8a	4.0b	9.2a	4.6b
1,3-bis(1,1-Dimethylethyl)benzene	230.6b	324.7b	338.5b	566.1a	355.6b	493.6a	319.0b
2-Phenylethanol§	4044.3b	3941.5bc	3530.8c	4770.5a	3682.0bc	4890.2a	3574.4c
Styrene§	107.9ab	109.3ab	105.4ab	142.3a	68.6bc	138.3a	64.3c
o-Xylene	13.8ab	15.1ab	16.2ab	18.7a	12.3b	17.6ab	13.0b
p-Xylene	10.8bc	9.9bc	10.9bc	16.9a	13.6ab	14.5ab	6.4c
Nor-isoprenoids							
β-Cyclocitral†	9.7a	8.5b	8.4b	6.4c	4.6d	5.3cd	4.5d
β-Ionone§	20.0a	17.7ab	15.9b	13.1c	6.9d	10.6c	6.9d
Others							
Heptadecane§	34.3a	14.7b	8.5c	7.4c	5.0c	5.8c	5.1c
Hexanal§	56.2b	61.8b	57.2b	119.3a	34.1b	100.5a	37.8b
Octanoic acid§	5198.4ab	5383.8a	5277.0a	5370.0a	4634.6b	4886.7ab	4587.1b

Values are the mean of n = 3 biological replicates. Values with different letters are significantly different ($P < 0.05$). †Increased abundance in one or more Shiraz Girdle wines; ‡decreased abundance in one or more Shiraz Girdle wines; §no significant difference between Shiraz Girdle and Control wines (Böttcher *et al.,* 2018). dpg, days post-girdle.

The elevated concentration of two unsaturated aldehydes, (*E*)-2-heptenal and (*E*)-2-octenal, in Girdle wines when compared with Control wines at 30 dpg (Table 42) was another indicator of differences in oxidative stress conditions between Girdle and Control berries, since both aldehydes represent volatile lipid oxidation products derived from linoleic acid (Frankel 1983). The increased oxidative degradation of this fatty acid in Girdle berries might have reduced its availability as a substrate for the lipoxygenase pathway (Schwab *et al.,* 2008) and could therefore also explain the relatively higher concentration of 1-hexanol, a product of the lipoxygenase pathway, in 20 and 30 dpg Control wines (Table 42).

In further support of oxidative stress as the likely trigger for changes in the volatile composition of Girdle versus Control wines, carotenoid cleavage enzymes are induced under oxidative stress (Mathieu *et al.,* 2005) and might have stimulated carotenoid breakdown and thus increased the concentration of three C_{13}-norisoprenoids (vitispirane,1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and *β*-damascenone) in girdled berries (Table 42). All three compounds contribute to the fruity aroma of Riesling (Chisholm *et al.,* 1994; Skinkis *et al.,* 2008) and vitispirane and *β*-damascenone have previously been described as increased in abundance in wines made from girdled Shiraz berries (this report). At higher concentration, TDN can impart an undesirable kerosene-like aroma to Riesling wines (Sacks *et al.,* 2012).

The depression of wine volatile formation by girdling was mainly transient, with 14 compounds, mostly esters and higher alcohols, found at increased concentration in Control wines at 20 dpg only (Table 42). It has previously been reported for Chardonnay wines, that the formation of both of these compound groups during the fermentation process is affected by the YAN source, correlating positively with the availability of α-amino acid nitrogen (Torrea *et al.,* 2011). The reduced formation of esters and higher alcohols in Girdle wines might therefore be the result of significantly depleted concentration of amino acids in Girdle berries (Table 41). Interestingly, despite an identical differential in total amino acid

concentration between Control and Girdle berries at 20 and 30 dpg (Table 41), only eight compounds, four of them higher alcohols, were found to be decreased in concentration in both 20 and 30 dpg Girdle wines, compared with that of the Control wines. Since the formation of wine volatiles such as higher alcohols, for example 2-phenylethanol, can depend on the abundance of specific amino acids, for example phenylalanine, subtle changes in amino acid ratios between the 20 and 30 dpg sampling time points might explain this observation. In addition, other phloem-imported compounds not considered in this study might be responsible for changed grape and/or yeast metabolism in 20 and 30 dpg berries and wines. A further eight wine volatiles, mainly esters and higher alcohols, were increased in abundance in Control wines compared with Girdle wines at all three sampling time-points (Table 42). Among them, ethyl 2-furoate has been shown to be reliably reduced in concentration in wines made from girdled or ripening-delayed berries (Böttcher *et al.,* 2012; Davies *et al.,* 2015; this report), consistent with its positive correlation with berry sugar content (Boss *et al.,* 2014).

6.14.4 Conclusions

Oxidative stress and restricted amino acid import, not sugar accumulation, were identified as possible elicitors for the increased accumulation of varietal wine aroma compounds in Riesling. Peduncle-girdling of post-veraison Riesling bunches had two main effects on wine volatile formation: a significant increase in monoterpenoid accumulation, evident from 20 dpg and persisting to 30 dpg and a more transient reduction in esters and higher alcohols, with the lowest concentration at 20 dpg. While elevated monoterpenoid concentration was indicative of an oxidative stress response, as supported by reduced berry Pro and iP concentration, the repression of the formation of esters and higher alcohols was likely due to the restricted import of amino acids into the berries. These findings suggest that berry amino acid quantity and composition, and iP accumulation in grapes during the later stages of rapid sugar accumulation, may represent potential targets for vineyard management strategies and grapevine breeding to influence the wine volatile profile of aromatic cultivars.

6.15 Partial girdling as a tool to study the relationship between sugar accumulation, time-on-vine and wine composition

6.15.1 Introduction

This study aimed to develop another method to investigate the nexus between berry sugar content, time-on-vine and wine composition. It can be technically difficult to separate the relative influences on grape metabolism and wine character of time-on-vine and sugar accumulation, but previous work has shown that the concentration of some wine volatile flavour and aroma compounds is influenced more by time-on-vine and that of others is influenced more by sugar content (Boss *et al.,* 2014). In this experiment instead of fully girdling bunch peduncles only partial girdles were performed. Partial peduncle girdling offers a more flexible approach than entire girdling as phloem transport can be reduced without preventing it entirely, thus providing a tool to manipulate sugar accumulation (and the flow of other phloem-transported molecules) to further study the relationship between berry time-on-vine, sugar accumulation and berry and wine composition. This experiment used partial peduncle girdling to study the interaction between sugar accumulation and time-on-vine in relation to berry and wine composition. Knowledge of their respective influences on wine outcomes should be useful in predicting and managing the effects of climate change.

6.15.2 Materials and methods

Shiraz bunch peduncles were partially girdled after veraison, which delayed development as measured by TSS and colour increase. Wines were made from non-girdled (Control) bunches and partially girdled bunches harvested at the same time as the Control (G1) and later, at the same TSS level as the Control (G2).

Girdling field experiment and berry analysis

Vitis vinifera L. cv. Shiraz vines grown on own roots and oriented in a North-South direction at Willunga, South Australia (Chalk Hill Wines, lat. 35°15'46.8"S; long. 138°33'10.8"E) were used for this study. Peduncle girdling was carried out three weeks post-veraison when the mean TSS, of berries from the three Control replicates was 15.8 ˚Brix. The Control treatment consisted of ungirdled bunches. For the girdled bunches, a 4-5 mm wide section of the phloem was removed from half the circumference of the peduncle so that the cross-section of the remaining phloem was approximately half that of the non-girdled Control. Both treatments were conducted in triplicate, 100 bunches per replicate for the Control treatment and 200 bunches for each replicate of the girdled treatment, with one replicate of each in three adjacent rows. In addition to the initial sample (0 days), samples were taken at 14, 21, 28 and 35 days post-girdling (dpg). Sixty berries from each replicate were deseeded immediately and the resulting skin and flesh were frozen in liquid nitrogen, transported on dry ice and stored at -80^{o}C for further analysis.

Winemaking

Fruit for winemaking was harvested on two dates. The Control treatment was harvested on 17 Feb 2015 (28 days after girdling) when the TSS measured in the three 60 berry replicate samples was 27.2 ^{o}Brix on average. One half of the bunches for each replicate of the partially girdled treatment was harvested at the same time (G1) as the Control bunches, the average TSS level for these berries was 23.7 ^{o}Brix. The second half of the gridled fruit was harvested on 24 Feb (G2, 35 dpg) at an average TSS level of 26.9 ^{o}Brix. Small-scale winemaking of the harvested berries and the analysis of basic wine parameters was conducted by WIC Winemaking Services (Urrbrae, SA, Australia).

Wine analysis

Sensory analysis was conducted via a triangle difference test (ISO 4120:2004). The consumer panel consisted of 36 members. One sequence of tests was completed using the standard clear glasses (ISO 3591:1977; http://www.iso.org/iso/catalogue_detail.htm?csnumber=9002). The second sequence was conducted in the same manner but used black 'blind tasting' glasses (http://www.barstuff.de/blindtasting-glaser-inao-rona-210ml.html) under red light conditions to counteract any of influence wine colour on flavour/aroma perception. The number of correct responses (when the different one of the three samples was correctly identified) was determined and compared against the critical value required for significant differences.

6.15.3 Results and discussion

Partial peduncle girdling delayed, but did not prevent, sugar accumulation in berries (Figure 79a). The concentration of TSS was significantly lower in partially girdled fruit than Control fruit from the first sampling time-point after girdling and remained so until the time of harvest of the Control fruit (G1). The harvest of G2 fruit was completed one week after that of the Control and G1 fruit, when grapes derived from partially girdled bunches had reached a similar TSS level to that of the Control grapes harvested a week prior. The rate of TSS increase was similar in Control and girdled fruit between 2 and 3 weeks post girdling and the rate was a little lower for gridled fruit between 3 and 4 weeks post girdling, but much of the delay in sugar accumulation occurred during the first two weeks after girdling (Figure 79a). This would suggest that there may have been some compensation of the flow rate through the remaining phloem following partial girdling of the peduncle. The accumulation of anthocyanins was also delayed by the girdling procedure. G1 fruit had significantly less anthocyanin content than the Control at the time of harvest, but the levels in Control and G2 fruit were statistically similar (Figure 79b). The interruption of the ripening process by partial girdling three weeks after veraison resulted in a statistically significant (ANOVA, $P > 0.05$) reduction in total acid content in G2 fruit (Control 4.53, G1 4.60, G2 3.40 g/L) and higher pH (Control 3.54, G1 3.46, G2 3.91). Presumably, this resulted from extending the time from veraison to harvest as organic acid concentration increases up until veraison and then decreases throughout ripening, due to berry expansion and the catabolism of mainly malic acid (Coombe 1992).

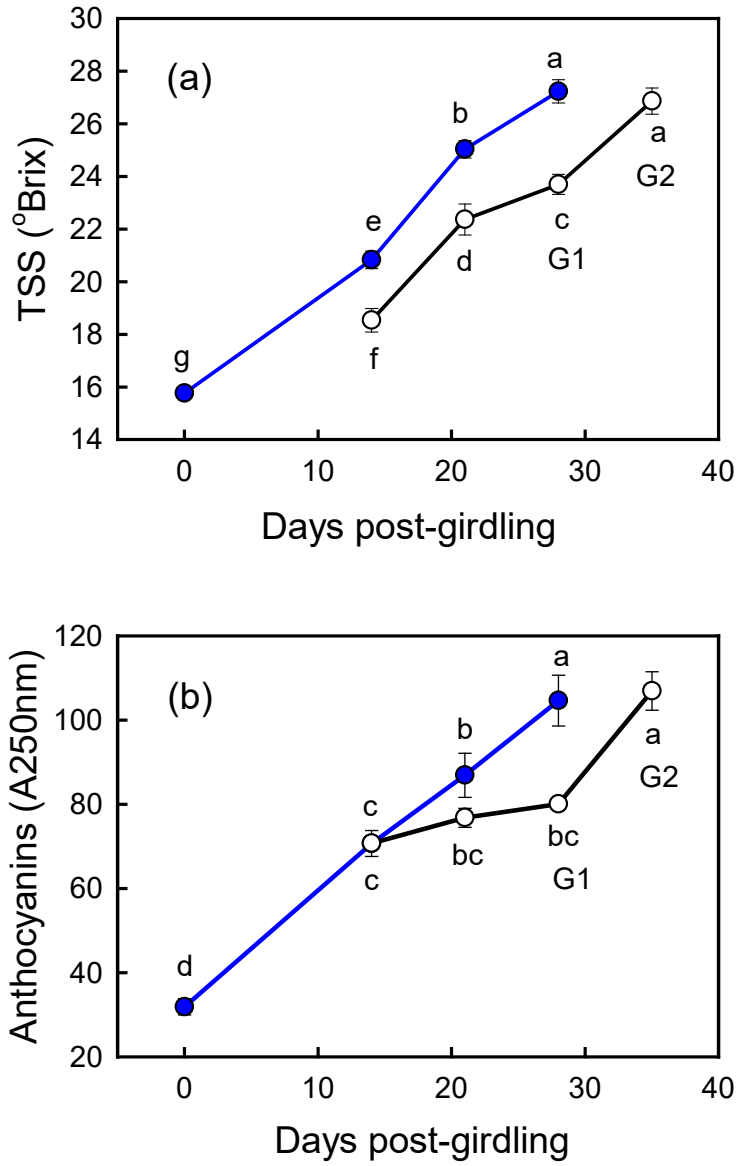


Figure 79. (a) Total soluble solids and (b) anthocyanin (A520nm) content, of berries from Control (blue) and partially girdled (open circle) bunches during ripening. G1 refers to the girdled fruit harvested at the same time as the Control fruit, G2 refers to the girdled fruit harvested at a later date than the Control fruit, but at approximately the same TSS level. Values represent means ± SE (*n* = 3) and are denoted by a different letter if the means differ significantly ($P < 0.05$) using one-way ANOVA followed by Duncan's post hoc test.

Partial girdling did not greatly alter the accumulation of a range of elements (major cations, trace elements and non-metallic elements; Figure 80). The concentration of calcium and zinc did not change significantly throughout berry development and there were no significant differences between the profiles of fruit from the Control, G1 and G2 bunches. The levels of boron, magnesium, phosphorus and sulfur tended to increase with time from the first sample point but there were no significant differences between the treatments at the same sampling time. The concentration of copper was lower in berries of the partial girdle treatments at 28 dpg (G1) and 35 dpg (G2) than in the Control at 28 dpg, whereas iron was at a higher concentration in the partial girdle sample at 35 dpg (G2). The concentrations of iron, and particularly copper, were quite low and the small differences in concentration between the treatments

may not greatly affect berry metabolism. The concentration of potassium was lower in the partial girdle, G1, fruit compared to the Control, but not significantly different in G2 grapes, when the TSS level was more similar to that of the Control. Although potassium can move in both xylem and phloem, its concentration in grape phloem is much higher (Rogiers *et al.,* 2017) and so a more dramatic difference in concentration between Control and berries from girdled bunches might have been expected. Potassium is known to be important to a range of processes during berry development (Rogiers *et al.,* 2017) and so the maintenance its concentration at harvest in ripening delayed fruit is another indicator that the ripening process in these berries is delayed rather than significantly altered. As described above with regard to sugar accumulation, there may have been some adaptation by the remaining phloem after the initial girdling event which compensated to some extent for the removed phloem tissue and so allowed the normal accumulation of potassium in girdled fruit.

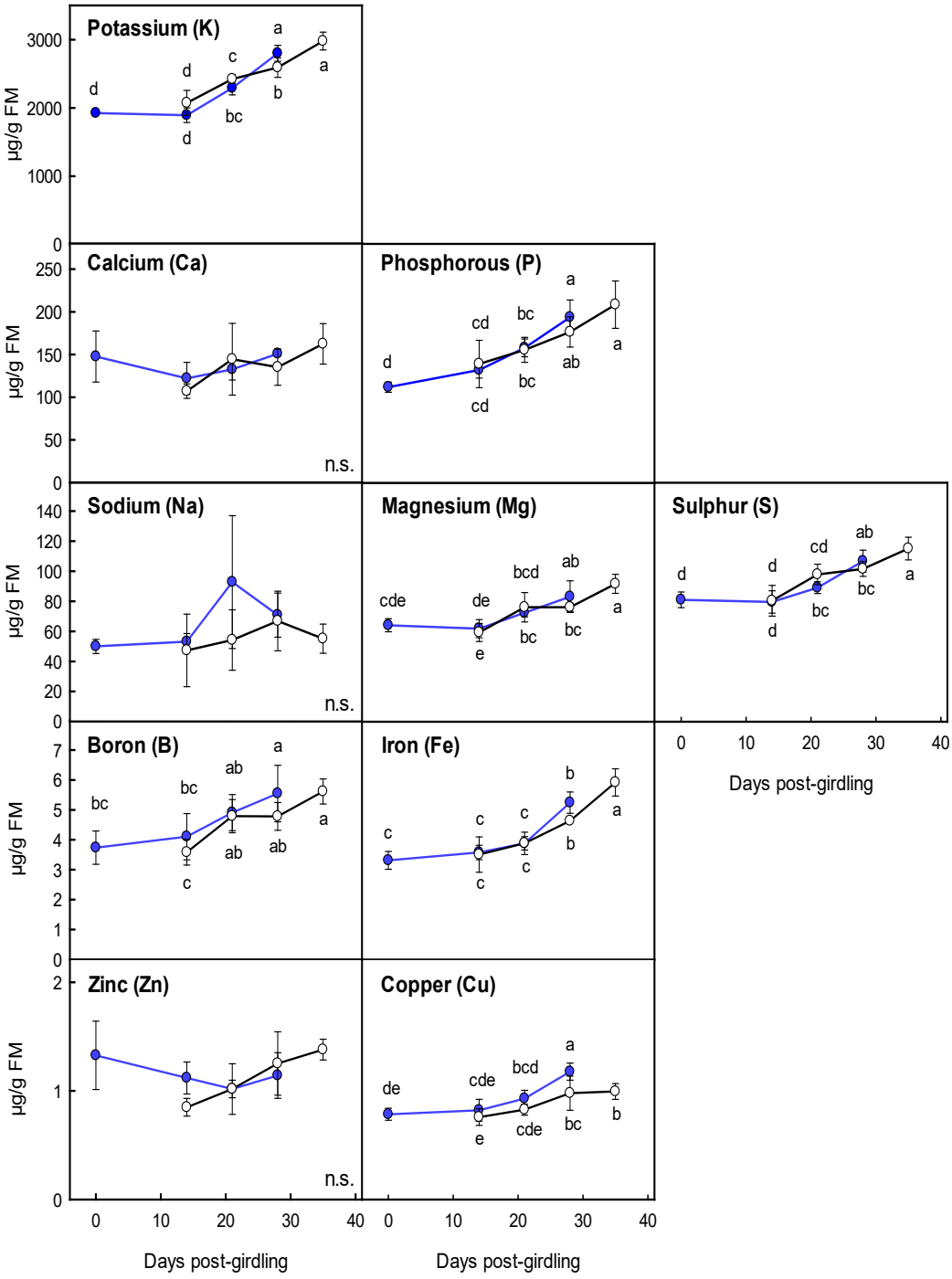


Figure 80. Concentration (µg/g fresh weight) of elements in fruit from control (solid circle) and half-girdled (open circle) bunches during ripening. The letters indicate statistical significance of the means (n = 3) as determined by ANOVA analysis followed by Duncan's post hoc test, $P < 0.05$, 'n.s.' indicates no significant difference.

As expected from the TSS data shown in Figure 79a, the wine made from G1 grapes had a significantly lower average alcohol content than the wine made from the Control grapes (Table 43). The average alcohol content of the wine made from the G2 grapes was not significantly different from the Control wine. Most of the other wine parameters were similar but the titratable acidity (TA), measured at pH 8.2, of G1 wine was lower than that for either the Control or G2 wines and the TA measured at pH 7 was lower for both of the wines derived from girdled fruit. The reduced wine TA and increased pH mirrors the differences in total acid content and pH in the harvested fruit described above.

Table 43. Wine bottling biochemical data.

Sample	Alc (% v/v)	Glc Fru (g/L)	SG	pH	TA at pH 8.2 (g/L)	TA at pH 7.0 (g/L)	FSO_2 (ppm)	TSO_2 (ppm)	VA (g/L)
Control	16.27*a*	0.80	0.9909	3.64	6.97*a*	6.43*a*	27.3	69.3	0.30
G1	14.17*b*	0.53	0.9908	3.57	6.00*b*	5.70*b*	28.3	79.0	0.43
G2	15.33*ab*	0.37	0.9918	3.66	6.57*a*	6.03*b*	30.3	68.7	0.30
		n.s.	n.s.	n.s.			n.s.	n.s.	n.s.

The means of three replicates are given for each of the three treatments for alcohol (Alc % v/v), glucose and fructose (Glc and Fru g/L), specific gravity (SG), pH, titratable acidity at pH 8.2 (TA at 8.2 g/L), titratable acidity at pH 7 (TA at pH 7.0 g/L), free sulfur dioxide (FSO_2 ppm), total sulfur dioxide (TSO_2 ppm) and volatile acidity (VA g/L). Letters indicate statistical significance as determined by ANOVA analysis followed by Duncan's post hoc test, $P < 0.05$, 'n.s.' indicates no significant difference.

The results of the GC-MS analysis of wine volatile compounds are shown in Table 44. In total there were 17 identifiable compounds that varied significantly in concentration between Control and G1 or G2 wines. Seven compounds differed in concentration between Control and G1 wines, three were at a higher concentration in the G1 wine, four were higher in the Control wine. Two of the four volatile compounds at a higher concentration in the Control wine compared to G1 wine, ethyl phenyl acetate and γ-butyrolactone, were also higher in the Control compared to G2 wine. Twelve, identified compounds differed in concentration between Control and G2 wines, nine were higher in Control, three were higher in G2 wines. Five esters, three of which were described as 'sweet, fruity or floral' were at a higher concentration in the Control than in G2 wines. Overall, only a small number of volatiles differed significantly between Control and G1/G2 wines and none of the differences in concentration were greater than 1.9-fold.

Table 44. Volatile compounds found to be significantly different in the headspace of the wines produced from half-girdled bunches at two stages (G1, G2) and Control bunches.

Compound	Control	Girdle 1	More abundant in	Fold change	Compound class	LRI ‡	ID§	Aroma character
1-Butanol	0.163†	0.096	Control	1.70	Alcohol	1154	A	Fusel
Hotrienol	0.007	0.010	Girdle	-1.35	Alcohol	1575	B	Tropical
3-Methyl-1-pentanol	0.303	0.207	Control	1.46	Alcohol	1306	A	Fusel
Benzaldehyde	0.171	0.320	Girdle	-1.89	Aldehyde	1497	A	Almond
Ethyl phenyl acetate	0.078	0.064	Control	1.21	Ester	1739	A	Sweet, floral
Methyl salicylate	0.007	0.010	Girdle	-1.37	Ester	1730	A	Wintergreen
γ-Butyrolactone	0.058	0.037	Control	1.56	Lactone	1594	A	Fatty
	Control	**Girdle 2**						
Benzyl alcohol	0.026	0.035	Girdle	-1.33	Alcohol	1820	A	Floral, balsamic
(Z)-3-Hexen-1-ol	0.303	0.196	Control	1.55	Alcohol	1361	A	Green
Isoamyl alcohol††	9.217	7.876	Control	1.17	Alcohol	1200	A	Fusel
2-Methyl-1-propanol	0.199	0.167	Control	1.19	Alcohol	1110	A	Ethereal
Ethyl-(*E*)-3-hexenoate	0.113	0.071	Control	1.60	Ester	1286	A	Sweet, fruity
Ethyl-4-hydroxy butanoate	0.131	0.095	Control	1.39	Ester	1753	B	Caramel
Ethyl phenyl acetate	0.078	0.051	Control	1.54	Ester	1739	A	Sweet, floral
Heptyl acetate	0.007	0.013	Girdle	-1.82	Ester	1355	A	Green
Isoamyl propanoate	0.083	0.055	Control	1.49	Ester	1180	A	Sweet, fruity
γ-Butyrolactone	0.058	0.041	Control	1.42	Lactone	1594	A	Fatty
β-Citronellol	0.902	0.687	Control	1.31	Monoterpene	1718	A	Floral
α-Terpineol	0.605	0.795	Girdle	-1.32	Monoterpene	1654	A	Pine

† Values represent means (n = 3) of the areas under the peaks of a selected ion relative to the relevant internal standard. Significant differences between treatments were determined at $P < 0.05$ using one-way ANOVA followed by Duncan's post hoc test. ‡LRI (linear retention indices) calculated from retention of the compound relative to the retention of a series of n-alkanes (C_8-C_{26}); §A: Identity confirmed by matching weight spectra and LRI with that of authentic standards; B: Tentative assignment based upon comparison with weight spectral libraries and published LRIs. ††Compound quantified in the 1 in 100 dilution.

As rotundone is a volatile compound that is active at low concentrations but is important to the character of Shiraz wines, its presence was measured by a targeted method. The concentration of rotundone in wines made from Control, G1 and G2 grapes (1.21, 1.20 and 1.33 ng/L respectively) was low, well below the proposed sensory threshold of 16 ng/L (Wood *et al.,* 2008) and not significantly different between the treatments. It has been shown that NAA treatment both delays ripening and considerably increases rotundone levels (Davies *et al.,* 2015; this report). The lack of the induction of rotundone in fruit delayed in ripening by partial girdling of the bunch peduncle is consistent with NAA, rather than a delay in harvest timing, being the causal factor. The effect of NAA could be a direct one on the synthesis of rotundone or its precursor (α-guaiene), may be due to the extension of the pre-veraison period which allows an increase in precursor levels, or an extension of the later stage of ripening under cooler conditions which facilitates rotundone accumulation.

The majority (11) of the 17 identifiable volatile compounds, behaved in a similar way in berries derived from partially-girdled bunches (Table 44) as they did in berries originating from bunches that had been entirely girdled in previous experiments conducted in either Riesling (Böttcher *et al.,* 2019, this report) or Shiraz (Böttcher *et al.,* 2017, this report), i.e. those at a higher concentration in G1 or G2 wines in the current work were also at a higher concentration in wines from at least one of the experiments using berries from fully-girdled Riesling or Shiraz bunches and *vice versa*. As the number of volatile compounds that varied between Control and girdled treatments was small and the fold-differences were low, it

indicated that, despite differences in sampling timing and TSS, berry metabolism was not greatly affected by the girdling process.

It is interesting to compare the effects that other methods of delaying harvest might have on volatile composition as the different mechanisms of action of these treatments, e.g. NAA application, might be expected to have effects on wine volatile composition not related simply to those associated with delaying ripening. In experiments using NAA to delay ripening only minor differences in wine volatiles were observed in the wines from ripening-delayed fruit and the wines from Control fruit harvested at a similar TSS level (Böttcher *et al.,* 2011b; 2012b, Davies *et al.,* 2015). This again indicates that delaying ripening results in mainly subtle changes to berry metabolism and wine volatile profile. However, although both girdling and auxin treatment result in small changes in the concentration of a limited number of volatile compounds compared to the Control treatments, there is little commonality in the identity of the volatiles affected. This may be partly due to varietal differences as both Shiraz and Riesling were used and there is also the likelihood that seasonal variation may be involved, however, some of the differences in volatiles between wine from girdled and auxin-treated fruit may be specific to NAA treatment.

An informal tasting determined that the replicate 1 wines (see Table 45 for details of replicate wine biochemistry analyses) for each treatment were generally representative of all three replicates and these were used for sensory analysis. Initially, triangle tasting was conducted using clear glasses and involved 36 participants where the Control and G1 and the Control and G2 wines were compared. Control and G1 wines (harvested at the same time but different TSS) were determined to be significantly different as were the Control and G2 wines (harvested at different times but approximately the same TSS, $P < 0.05$). As the wines from the girdled berries were lighter in colour (A520 nm of replicate 1 Control, G1 and G2 wines 15.42, 11.73 and 14.94 respectively) it was possible that tasters were using colour as a cue to discriminate between the wines and so the sensory analysis was repeated using red lighting and black glasses. In this second sensory analysis, no significant differences ($P < 0.05$) were detected despite the G1 wine being 3.3% lower in alcohol concentration than the Control wine (Table 45).

Table 45. Wine bottling biochemical data.

Sample	Alc (% v/v)	Glc & Fru (g/L)	SG (g/L)	pH	TA at pH 8.2 (g/L)	TA at pH 7.0 (g/L)	FSO_2 (ppm)	TSO_2 (ppm)	VA (g/L)
Control 1	**16.1**	**0.2**	**0.9911**	**3.77**	**6.8**	**6.2**	**29**	**68**	**0.22**
Control 2	16.0	0.7	0.9912	3.62	7.2	6.7	26	67	0.31
Control 3	16.7	1.5	0.9905	3.54	6.9	6.4	27	73	0.37
Girdle 1-1	**13.4**	**0.2**	**0.9915**	**3.66**	**5.8**	**5.5**	**29**	**79**	**0.38**
Girdle 1-2	14.1	0.3	0.9907	3.60	5.9	5.7	25	68	0.42
Girdle 1-3	15.0	1.1	0.9902	3.46	6.3	5.9	31	90	0.50
Girdle 2-1	**14.3**	**0.6**	**0.9924**	**3.71**	**6.4**	**6.0**	**26**	**60**	**0.42**
Girdle 2-2	15.7	0.2	0.9913	3.69	6.6	6.0	33	71	0.22
Girdle 2-3	16.0	0.3	0.9916	3.59	6.7	6.1	32	75	0.25

The replicates used for sensory analysis are shown in bold type. Values are given for alcohol (Alc % v/v), glucose and fructose (Glc and Fru g/L), specific gravity (SG), pH, titratable acidity at pH 8.2 (TA at pH 8.2 g/L), titratable acidity at pH 7.0 (TA at pH 7.0 g/L), free sulfur dioxide (FSO_2 ppm), total sulfur dioxide (TSO_2 ppm) and volatile acidity (VA g/L).

6.15.4 Conclusions

The data presented show that girdling only part of the circumference of a bunch peduncle can be used to retard the rate of berry ripening, in contrast to the delay resulting from auxin application which shift veraison, i.e. delay entry into the ripening phase (Böttcher *et al.,* 2011, 2012; Davies *et al.,* 2015). Although

it is an intrusive technique, it is another tool that can be used to investigate the relationship between time-on-vine, sugar accumulation and the metabolism of flavour/aroma compounds and their precursors. Partial girdling enables the flow of sucrose and other molecules carried in the phloem to be reduced, but not stopped, so that berries ripen in a seemingly normal manner, but achieve the same TSS value as non-girdled fruit at a later date. Despite these two methods working through different mechanisms to control ripening they have similar outcomes. In general, modest delays in ripening do not appear to greatly affect either berry and wine composition or wine sensory properties. Therefore, a delay in ripening, to mitigate the effects of climate change on seasonal compression should, by itself, not be detrimental to wine quality.

6.16 Phenotyping the CSIRO First Generation mildew resistant lines and SARDI wine grape varietal collection for phenological and agronomic traits

6.16.1 Introduction

As mentioned in the Background section, it was decided that a broader approach to resolving some of the climate change-induced problems would be a prudent approach given the limitations in breeding novel cultivars and the use of agrochemicals/labour and fossil fuels to control ripening. The ultimate aim of the new strategy commenced in this project is to use phenotyping data for the identification of the causal genes for trait variation and to exploit this information to develop new clones of currently grown premium cultivars whose wines retain their original flavour/aroma profiles but have improved growth/ripening characteristics. The phenotyping for traits of interest needed to be done in a population that was readily available and contained sufficient variation, which was the case for the CSIRO first generation mildew resistant lines. The main traits to be targeted include those involved in determining harvest timing, such as the timing and length of budburst and flowering periods, the timing of veraison, the length of the period from end of flowering to veraison and the length of the period from veraison to harvest (largely the sugar accumulation rate). As final berry weight is an important agronomic trait, maximum berry weight and the degree of weight loss or gain after reaching 20 ^{o}Brix were also seen as potentially important. Additional traits such as the synchronicity of ripening between berries is seen as important along with other interacting characters such as canopy size and yield per canopy size. A major focus is the potential for developing clones with different harvest times. Shifting the harvest period by having cultivars with delayed budburst/flowering would have the additional benefit of making frost damage and reduced fruit set less likely.

Another trait that is important to the wine industry is the tolerance to Eutypa infection. Data regarding the susceptibility of a large number of cultivars to Eutypa was recently published (Sosnowski *et al.,* 2022) and these data will be used for mapping studies along with the traits mentioned above. Dr Sosnowski has offered help where needed but the first step is to determine if we can get a reasonable 'hit' through genetic mapping. This trait will be analysed along with the other traits except that the population is different as these data were gathered from the SARDI wine grape variety collection at Nuriootpa, SA. The data show that the varietal collection contains a considerable range of tolerance, from very few symptoms to arm or vine death. Unfortunately for the industry, a number of the cultivars most widely planted in Australia have relatively poor tolerance to infection e.g. Shiraz, Sauvignon Blanc and Cabernet Sauvignon.

The scoring of the other traits is a significant investment of time and there are some challenges.
As these traits are developmental in nature, the assessment of change will have to occur over quite a long period and will have to be done at very frequent intervals. In addition, while some measurements can be quite exact, as long as the sampling regime provides a representative sample, other characters, such as budburst timing, are measured via subjective assessment and can be much more difficult to determine.

As a precursor to determining the genetic cause of variation in phenological and agronomic traits, we needed to measure how much diversity there was in the available crosses for the range of traits we deemed potentially useful. These data will then be used for mapping studies where the first step will be to identify the region of the genome most likely to have a genetic variation that explains the phenotype in question and then focus in on the precise cause i.e. identifying the actual genetic difference, the functional effect and how this might be manipulated through gene editing.

To try to account for seasonal effects, data for the agronomic and phenological traits were collected over two successive seasons. Lessons regarding how to phenotype the traits from the first year were used to modify the methodology in the following season.

6.16.2 Materials and Methods

6.16.2.1 Eutypa susceptibility – SARDI varietal collection

Data regarding the susceptibility of the cultivars in the SARDI collection were kindly supplied by Dr Mark Sosnowski (SARDI). These data are being used for mapping the loci responsible for tolerance/susceptibility to Eutypa by GWAS (genome wide association study) using the SNP markers previously developed for this population by Dr Boss's group.

6.16.2.2 Phenological data for CSIRO First Generation lines at SARDI, Nuriootpa

The budburst timing and flowering of 131 first generation mildew resistant lines was visually assessed. An approximate measure of the percentage of buds and flowers at the different stages was recorded. To ensure samples for measurements of weight and Brix were taken at veraison (10 ^{o}Brix was used as the target for the time of veraison to try to account for inaccuracies due to limited sampling size and possible berry heterogeneity) and 20 ^{o}Brix small-scale samples from each line were taken as required when veraison and 20 ^{o}Brix appeared to approaching. The Brix from juice from these berries was measured with a hand-held refractometer in the field. When required, 60 individual berries were sampled, transported to the laboratory on ice and Brix and berry weight were measured for each berry. Sampling was also conducted at 20 ^{o}Brix plus two weeks (2019/20) and 20 ^{o}Brix plus three weeks (2020/21) and berry Brix and weight measured as above. Yield was measured by weighing all the harvested fruit from each line (six vines per panel, one panel per line) at the time of the last sample (20 ^{o}Brix plus two weeks 2019/20; 20 ^{o}Brix plus three weeks 2020/21). Pruning weight was used as the measure of vine vigour in the 2019/20 season. Vines were pruned to 20 spurs each with two buds, the pruned canes were collected and weighed in the field.

During the 2020/21 season, vine vigour was measured as canopy coverage by Dr Betti (CSIRO) using drone imaging, which was then processed to give a %coverage. Several aerial images of the experimental block were taken with a DJI Phantom 4Pro drone flying at the height of 30 m. Using photogrammetry software (Agisoft Metashape, AgiSoft PhotoScan Professional Version 1.8 (2021) retrieved from http://www.agisoft.com/), the images were collated to create an RGB georeferenced ortho-mosaic, a digital surface model (DSM) and a digital terrain model (DTM, surface map without above-ground objects) of the entire block. A binary georeferenced image representing the green canopy cover of the grapevines (GCC) was created using the raster calculator tool in QGIS (QGIS Development Team (2021) (QGIS Geographic Information System, Open Source Geospatial Foundation Project. http://qgis.osgeo.org)) by classifying the pixels belonging to the ground (pixel value= 1) and those belonging to the grapevine canopy (pixel value = 1) using the following formula: (DSM – DTM)>0.6, where 0.6 was the arbitrary minimum above-ground height (m) for pixels belonging to the grapevine canopy. The mean GCC of vines within individual panels in each row of the experimental block was then calculated using QGIS to create a colourised map.

Structure of population

There are 131 lines, which were generated through crossing the female parent 'R23' which contains the mildew resistance loci with 11 different male parents (Table 46). R23 is a complex cross containing 50% Cabernet Sauvignon, 25% Aubun, 12.5% Merlot, 6.25% Grenache, 3.12% Cabernet Sauvignon, 1.56% Malaga with 1.56 % *M. rotundifolia* (the total Cabernet Sauvignon content is therefore 53.12%).

Table 46. Male parents and number of progeny for the first generation mildew resistant lines.

Male parent	Number of lines
Chardonnay	5
Muscadelle	2
Muscat Gordo Blanco	17
Verdelho	5
Frontignac	25
Dunkelfelder	36
Tempranillo	1
Tannat	14
Shiraz	2
Sangiovese	1
Riesling	23

The vines were grown at the SARDI Research Station at Nuriootpa, SA. Each line was represented by six vines. Vines were pruned to 20 shoots, 40 buds.

6.16.2 Results and discussion

Phenological data for CSIRO First Generation lines at SARDI, Nuriootpa

The amount of data collected is extremely large and diverse and it would be impossible to present all of the data in this report. Instead, there is an overall summary and some examples of the types of data collected and how the data could be used in the future to map genomic regions responsible for desirable traits with the aim of using gene editing to produce elite cultivars with additional desirable traits.

6.16.2.1 Season 1 – 2019/20

A wide range of traits were surveyed for each of the 131 lines, these included:

- Timing of initiation, and duration of, flowering
- Time of veraison
- Time of flowering to veraison
- Time of veraison to 20 ^{o}Brix
- Time of flowering to 20 ^{o}Brix
- Date of 20 ^{o}Brix
- Rate of Brix increase (sugar accumulation) between veraison to 20 ^{o}Brix
- Rate of Brix increase between 20 ^{o}Brix and 20 ^{o}Brix +2 weeks (late ripening period)
- Rate of weight change from 20 ^{o}B to 20 ^{o}B+2 weeks
- Mean berry weight
- Yield
- Pruning weight

- Yield per pruning weight
- Berry weight %SD at different stages
- Brix (TSS) %SD at different stages

Veraison was defined as 10 °Brix, simply because in the very early stages of sugar accumulation Brix (TSS) increases very rapidly and there can be a high degree of asynchronous development. 20 °Brix was chosen as a crucial stage as it is around the time when active sugar uptake into berries slows down considerably or stops. From this stage onwards, Brix increase is slowed and can be, at least in part, due to berry dehydration which results in solute concentration within the berry.

From the data obtained through in-field observation/measurement and in-lab measurement for the various traits, listed above, the range of values for many important traits can be calculated that show the variation available in the lines used in this study. Obviously, flowering date is an important time-point in determining the time of berry development and ultimately harvest timing, although as we will observe, the timing of other events is also important. The end of flowering date was 41 to 66 days after 1 Oct. The range of 25 days shows that there is sufficient genetic variability available to change the flowering date by about four weeks. This is a significant period and if translated into elite cultivars, it might assist in managing cold damage in cooler areas and if all else was equal, would significantly expand the range of harvest timing.

The range in the times taken for three other crucial stages is given in Table 47. As with the time of flowering there were considerable, even broader ranges, in the times for flowering to veraison, veraison to 20 °Brix and flowering to 20 °Brix. The large standard deviation values reflect the considerable variation seen. Although the differences in these ranges (up to 41 days) will not help with avoiding early season low temperatures they are valuable in achieving the main aim of diversifying the length of the harvest period.

Table 47. Timing of phenological stages from flowering to 20 °Brix

	Time end of flowering to Veraison	**Time Veraison to 20 °Brix**	**Time end of flowering to 20 °Brix**
Mean (days)	70.44	28.76	99.20
Median (days)	71	27	98
Lowest-highest	55-88	13-54	84-125
Range (days)	33	41	41
SD	5.76	8.68	7.44

The relative duration of the two very important developmental phases, the time taken to progress from the end of flowering to veraison and the time to progress from veraison to 20 °Brix can be visualised as a plot of the ratio of these traits Figure 81). It can be seen from the plot that in some lines, the time for both phases was similar but for the large majority of lines the time from flowering to veraison (10 °Brix) is much longer than for veraison to 20 °Brix. Again, this shows that there is great deal of potential variation that could be tapped to manipulate the timing of harvest.

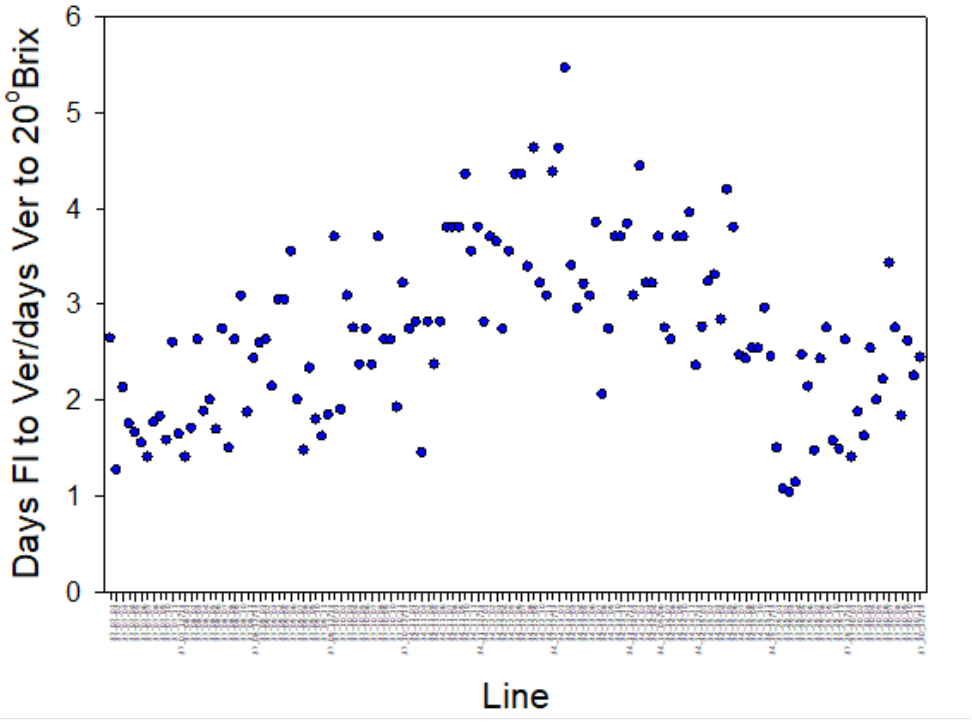


Figure 81. Ratio of time of flowering to veraison/time of veraison to 20 °Brix for individual lines.

Another way of describing the 'speed' of ripening is by calculating the rate of Brix change from veraison to 20 °Brix (°Brix/day), which varied from 0.192 to 0.7143 (Figure 82). The calendar date for reaching 20 °Brix is also a measure relevant to altering the time of harvest. The variation observed, determined as days after 1 February, was from 5-47 days which is a range of 42 days.

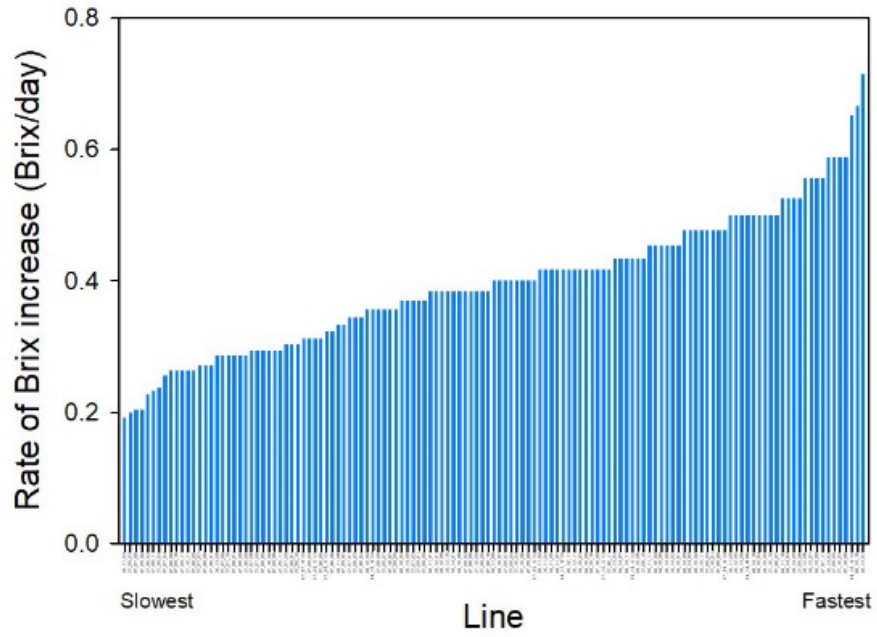


Figure 82. Rate of Brix increase (veraison to 20 °Brix) for individual lines.

Berry weight can also be a character of interest to growers and winemakers and can influence bunch openness. In the lines measured, the mean berry weight at 20 °Brix ranged from 0.41-1.36 g. This wide variation is not surprising, given the diversity of the genetic backgrounds of the parents.

Two other traits of interest involved the amount of variation in berry weight and Brix as measured by their percent standard deviation (Figure 83). The %SD for berry weight measured at 20 °Brix ranged from 17.95%-38.07% which shows that in some lines there is a large variation in size. Some of this can be attributed to a tendency for some lines to produce 'hen and chicken' bunches (Figure 84). However, the number of lines that present like this do not account for all of the variation measured. Interestingly, the range of %SD of Brix at 20 °Brix is much less, 3.04%-17.53%. Again, this is quite a range and suggests that there are lines within this population with a genetic background that produce fruit that is much less homogeneous that others. This has the potential to be a very important character as although some variation may be tolerable, or even desirable, in order to contribute to wine complexity, too much can be problematic. There is evidence that the Brix level can influence wine character (Boss *et al.,* 2018). A mixture of low sugar, under-ripe and high sugar over-ripe berries may contribute unwanted 'green' and 'cooked' characters respectively.

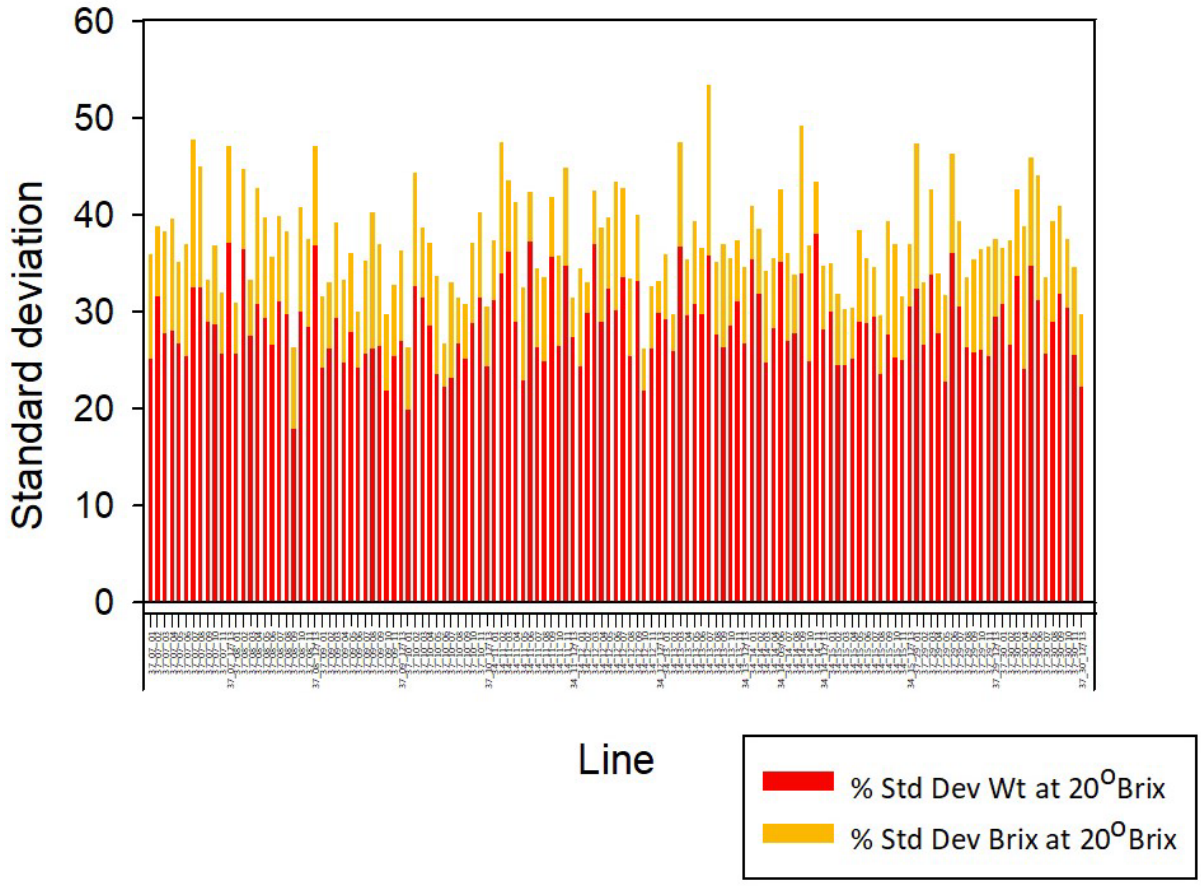


Figure 83. Stacked bar chart for %SD weight and %SD Brix at 20 °Brix.



Figure 84. Line producing 'hen and chicken' bunches.

The relationship between the weight and Brix of berries is quite complex and dependent on genetics as is shown by the two figures below (Figures 85 and 86). Sixty berries from two lines from the same cross were taken at three stages of development (veraison, 20 ^{o}Brix, and 20 ^{o}Brix plus two weeks) and the weight and Brix were measured for each berry. In both cases, the veraison berries showed a wide diversity in Brix values (SD = 2.19 and 2.02 for lines 34-11-06 and 34-11-07 and respectively), which is expected as we know that the initiation into the ripening phase is not particularly synchronous. The Brix values show less diversity as harvest approaches, for example when the mean Brix for Line 34-11-06 (Figure 85) reached 21.03 (red symbols) the SD was 1.07, for Line 34-11-07 (Figure 86) at 20.71 mean Brix (red symbols) the SD was 1.69, significantly higher, indicating that this line has less synchronous ripening. The interesting point is that the SD for berry weight was very different as for Line 34-11-06 (Figure 85) it was 0.3 compared to 0.14 for line 34-11-07 (Figure 86). These differences in berry weight and TSS synchronicity between the two lines can be seen clearly when Figures 85 and 86 are compared. It can be proposed that the flexibility in berry growth in some lines enables the Brix values to be more synchronous. This phenomenon was also proposed as a mechanism involved in the delay of ripening by NAA that resulted in berry expansion and more synchronised Brix levels (see earlier in this document).

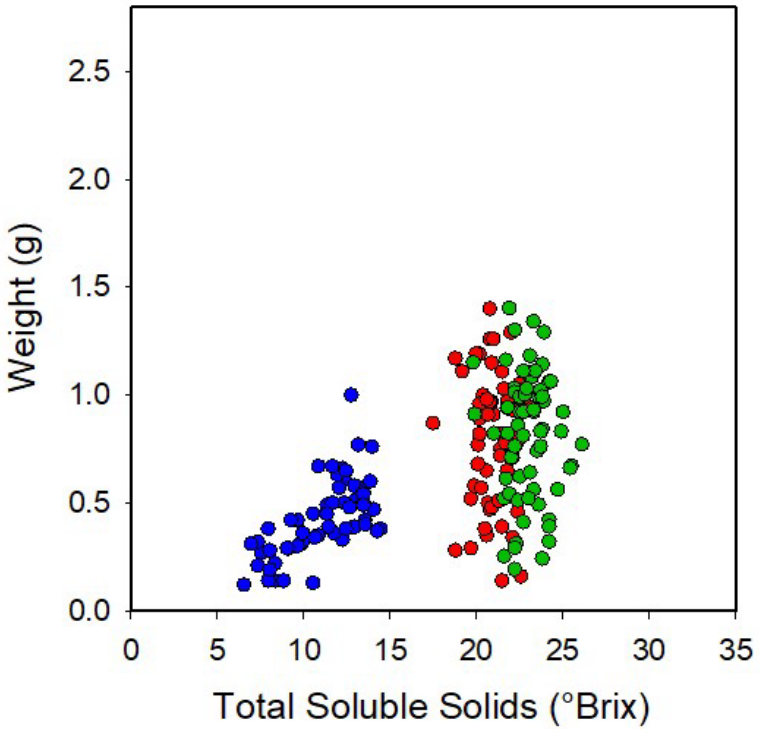


Figure 85. Berry weight vs TSS (°Brix) for line 34-11-06. Blue = berries at veraison, red = berries at an approximate mean of 20 °Brix, green = berries at 20 °Brix + 2wks.

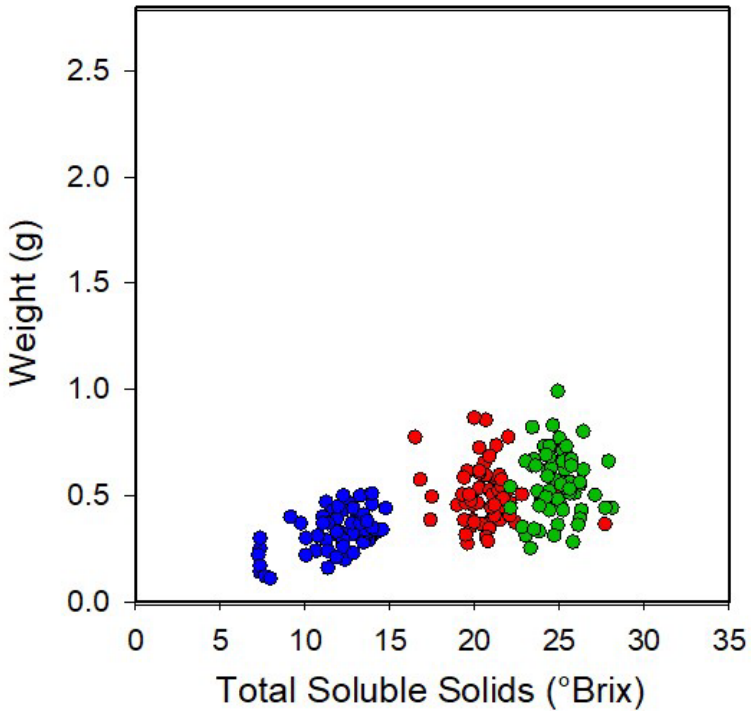


Figure 86. Berry weight vs TSS (°Brix) for line 34-11-07. Blue = berries at veraison, red = berries at an approximate mean of 20 °Brix, green = berries at 20 °Brix + 2wks.

The later stages of ripening/maturation are of interest particularly as some cultivars e.g. Shiraz can suffer weight loss and shrivelling. The ability to be able to select cultivars without this character or modify Shiraz

so that it does not undergo shrivelling would be a distinct advantage to industry. The data for the 2019/20 season showed that the rate of weight change from 20 °Brix to 20 °Brix+2 weeks varied significantly ranging from negative values (berry shrinkage) of -0.0071 to positive values (berry expansion) of 0.0108 g per day. This is a conservative estimate of shrinkage as commercial practice would be to leave the fruit on the vine for a longer period. During the same period the rate of TSS increase also varied quite a lot (0.012-0.489 °Brix per day), this rate is affected by both the change in berry volume but also possible variation in the rate of sugar loading into the better at the later stages of development.

Total yield is always an important consideration and the yield per vine varied considerably between the lines, from 0.96 to 9.61 kg/vine. Cane pruning weight is an accepted estimate of canopy size and like yield per vine varied considerably, from 0.095 to 1.593 kg/vine. When considered together, the yield per pruning weight ratio exhibited a very large range, from 1.42 to 37.50 kg/kg. Although not considered further here, this may have important implications for water use and photosynthetic efficiency. A plot of yield per panel ordered by berry yield per vine (Figure 87) demonstrates that the yield was not correlated to pruning weight. The reasons for this may be related to the number of bunches, berry size, photosynthetic efficiency, root structure etc., but it does indicate that canopy size does not determine yield.

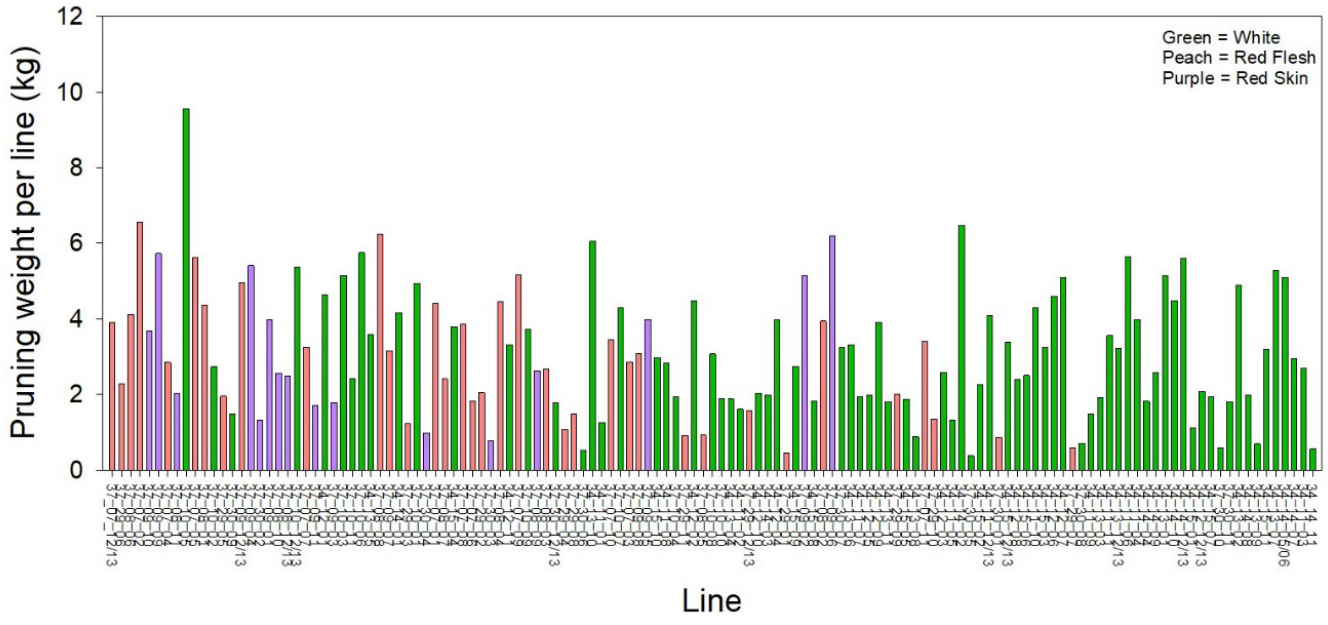


Figure 87. Pruning weight ordered according to yield (highest to lowest, left to right)

A considerable range of bunch openness was also observed in these lines (Figure 88).



A) **B)** **C)**

Figure 88. A) Line with compact bunches, B) C) lines having open bunch structure.

These data can be further interegated to bettter understand the relationships between the different parameters measured. The data was examined using Pearson's correlation as a measure of relatedness. A few examples are given here. Berry weight at different developmental stages correlated positively and significantly. The best correlation, not surprisingly, was between berry weight at 20 °Brix and at 20 °Brix+2 weeks, but the weight of berries at veraison for each line correlated well with their weight at 20 °Brix. Figure 89 shows that for any cultivar the final berry weight at harvest could be predicted by the weight at veraison.

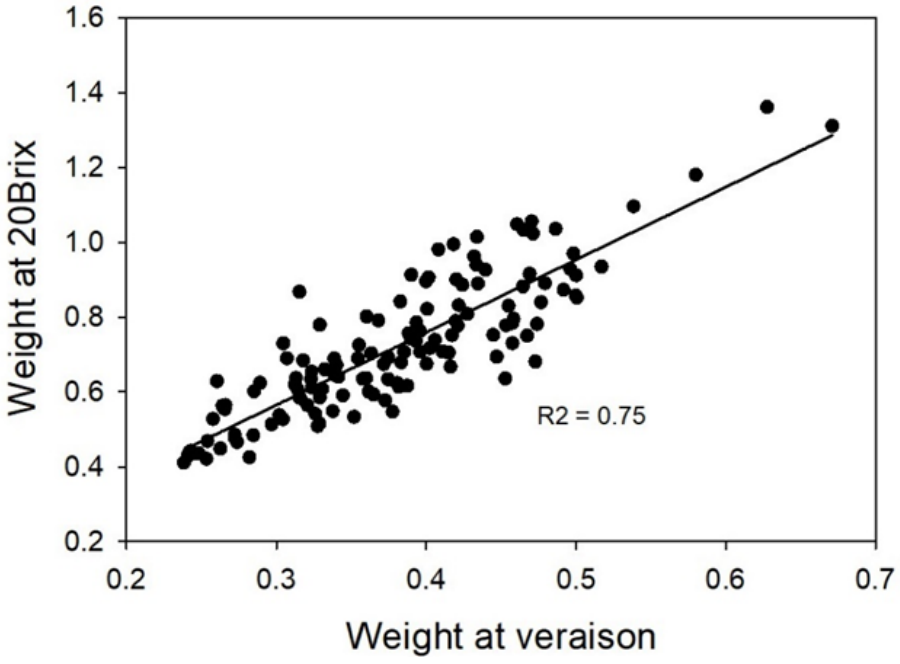


Figure 89. Relationship between weight at veraison and weight at 20 °Brix, data for all lines plotted, linear correlation.

The correlation between %SD for Brix at veraison and %SD for Brix at the 20 °Brix stage was poor. However, there was a good, positive correlation between the %SD for Brix at the 20 °Brix and 20 °Brix+2wks stages which indicates that the variance in Brix has settled down quite a lot by the 20 °Brix stage. There were also some positive correlations between the time taken to get from flowering to 20 °Brix, from veraison to 20 °Brix and veraison to 20 °Brix+2wks with %SD for Brix as the longer these developmental periods were the higher was the %SD for Brix. The same pattern occured for yield per vine. The longer these phases took, the higher the final yield.

6.16.2.2 Season 2 – 2020/21

The list of characters surveyed during the 2020/21 season was similar to that for the 2019/20 season. There were three main differences 1) the timing of bud development was included, 2) the post-20 °Brix sample was conducted at three weeks rather than two weeks post-20 °Brix, 3) remote sensing by drone overfly was used to determine the canopy coverage rather than using pruning weight as a proxy measure.

- Timing of bud development (% of dormant, swelling start, swelling end, woolly buds, green tip, bud opening)
- Timing of initiation and duration of flowering
- Time of veraison
- Time of flowering to veraison
- Time of veraison to 20 °Brix
- Time of flowering to 20 °Brix
- Date of 20 °Brix
- Rate of Brix increase (sugar accumulation) between veraison to 20 °Brix
- Rate of Brix increase between 20 °Brix and 20 °Brix +3 weeks (late ripening period)
- Rate of weight change from 20 °Brix to 20 °Brix+3 weeks
- Mean berry weight
- Yield
- %Canopy cover
- Yield per %canopy cover
- %SD of berry weight at different stages
- %SD of Brix at different stages

The absolute values (and to some extent ranges) for the various traits measured varied somewhat between the successive years, presumably due to seasonal differences. However, the patterns/trends for most traits were very similar for each line when compared across the years. This is highly encouraging, as it suggests that the traits we are looking at can vary with environment but they are primarily determined by the genetic makeup of the vines.

The data presented in Table 48 compare very favourably with those in Table 47. The means and medians are very similar for the three developmental stages. The ranges for ‘time from veraison to 20 °Brix’ are quite distinct but this is because there were a few outliers in the 2019/20 data that make the range larger in that season. This is also indicated by the SDs which were similar between the years for ‘time of flowering to veraison’ and ‘time of flowering to 20 °Brix’ but the SD for ‘time veraison to 20 °Brix’ 2019/20 was more than twice that for 2020/21 season. This suggests the traits being measured are governed more by genetics than seasonal differences.

Table 48. Timing of phenological stages from flowering to 20 °Brix

	Time end of flowering to Veraison	Time Veraison to 20 °Brix	Time end of flowering to 20 °Brix
Mean (days)	65.44	29.43	94.87
Median (days)	65	28	93
Lowest-highest	47-79	14-70	77-128
Range (days)	32	56	51
SD	6.03	7.75	7.46

Ranges of traits measured to show variation within the 'population':

- Time of 100% budburst as days after 1 Sep (10-31) Range of 21 days
- Flowering end date days after 31 Oct (9-24) Range of 15 days
- Days from end of flowering to veraison (47-79) Range 32 days (Table 48)
- Days from end of flowering to 20 °Brix (77-128) Range 51 days (Table 48)
- Days from veraison to 20 °Brix (14-70) Range of 56 days (Table 48)
- Time of 20 °Brix (days after 20 Jan) (18-60) Range of 42 days
- Rate of Brix increase veraison to 20 °Brix (0.165-0.644 °Brix/day)
- %SD of Brix at 20 °Brix (3.777547-15.08923)
- Mean berry weight at 20 °Brix (g) (0.54-2.03g)
- %SD of berry weight at 20 °Brix (17.58783-34.63985)
- Rate of Brix change between 20 °Brix and 20 °Brix +3 weeks (-0.014-0.376 °Brix/day)
- Rate of berry weight change from 20 °Brix to 20 °Brix+3 weeks (-0.0669 to 0.0212 g/day)
- Yield per vine (2.1-14.2 kg)
- %Canopy cover (4.637-47.50789)
- Yield per %canopy cover (58.9-535.2)

The additional trait measured in these lines in 2020/21 was budburst. The three-week period between 100% budburst for the early and later 'bursters' is a considerable period that should be useful in delaying not only budburst (possible frost avoidance, better pollination and set) but also in delaying the harvest period. The difference is shown dramatically in Figure 90 where the buds on the line in the foreground have just burst while those in the next panel have many shoots and leaves already.



Figure 90. Photograph showing the dramatic difference in the timing of budburst between phenotyped lines.

Instead of using the labour-intensive method of measuring pruning weights as a proxy for canopy size/vine vigour, we asked Dr Giacomo Betti from Dr Everard Edwards' group to use a drone to determine % canopy coverage. Figure 91 shows a section of the vineyard colour coded to show the estimated coverage. Note that only a range is given for each of the four, blue shadings but the full data set contained precise measurements.

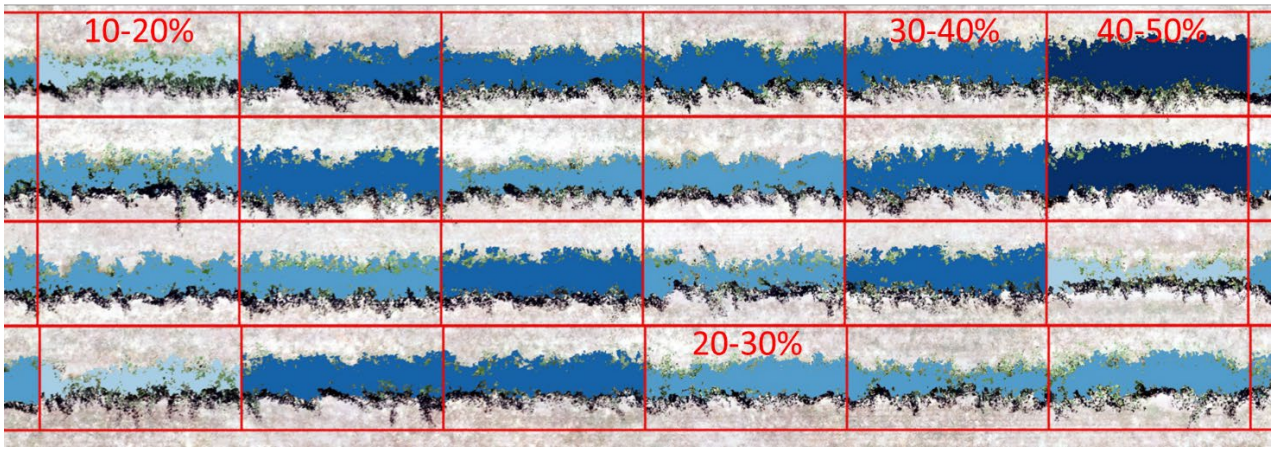


Figure 91. Drone data for canopy coverage (range % only shown – actual values were more precise).

The pruning weight data from 2019/20 when compared with canopy coverage assessed by drone from 2020/21 had a Pearson coefficient of 0.735. Given that there may have been some variation between the two seasons this correlation seems highly significant. The remote sensing method for determining canopy coverage may be the better measure as it may be more linear than pruning weight as an estimator, is a measure of leaf area and the data gathering is much quicker and less labour intensive than collecting pruning weight data.

Note that the Pearson correlation between budburst time and flowering time for 2020/21 was 0.822 which is highly significant, especially given that these two traits are quite hard to measure as both stage

of flowering and budburst has to be estimated for individual organs. It suggests that our assessments of budburst and flowering were quite accurate and that it is most likely that the timing of flowering is determined by the timing of budburst. This is not altogether surprising but does suggest that we should be targeting budburst rather than flowering when it comes to discovering the genes that control the timing of flowering.

As for the 2019/20 season (Figures 85, 86) different lines had quite different characteristics in terms of berry weight and TSS homogeneity. Figure 92 shows the TSS vs weight plots for berries from two lines compared at approximately 20 °Brix (mean). The blue symbols and means represent data from the 2020/21 season, the red, overlaid in the lower graph, data from the 2019/20 season. The first thing to note is that the distributions for each of the two lines was consistent over the two years (lower left and right panels) indicating that the patterns are stable and seem to be genetically controlled and not greatly influenced by season. It is also clear that the weight distribution was similar for both lines over both years. However, the line on the right is much more homogeneous in terms of TSS spread than the one on the left. This can be seen visually and is confirmed by the respective SDs. This means that the line on the right produces berries that are much more synchronous in terms of TSS/Brix which in turn may mean that they are also more synchronous in terms of wine flavour and aroma compounds and their precursors. This may be a distinct advantage in terms of wine composition and consistency.

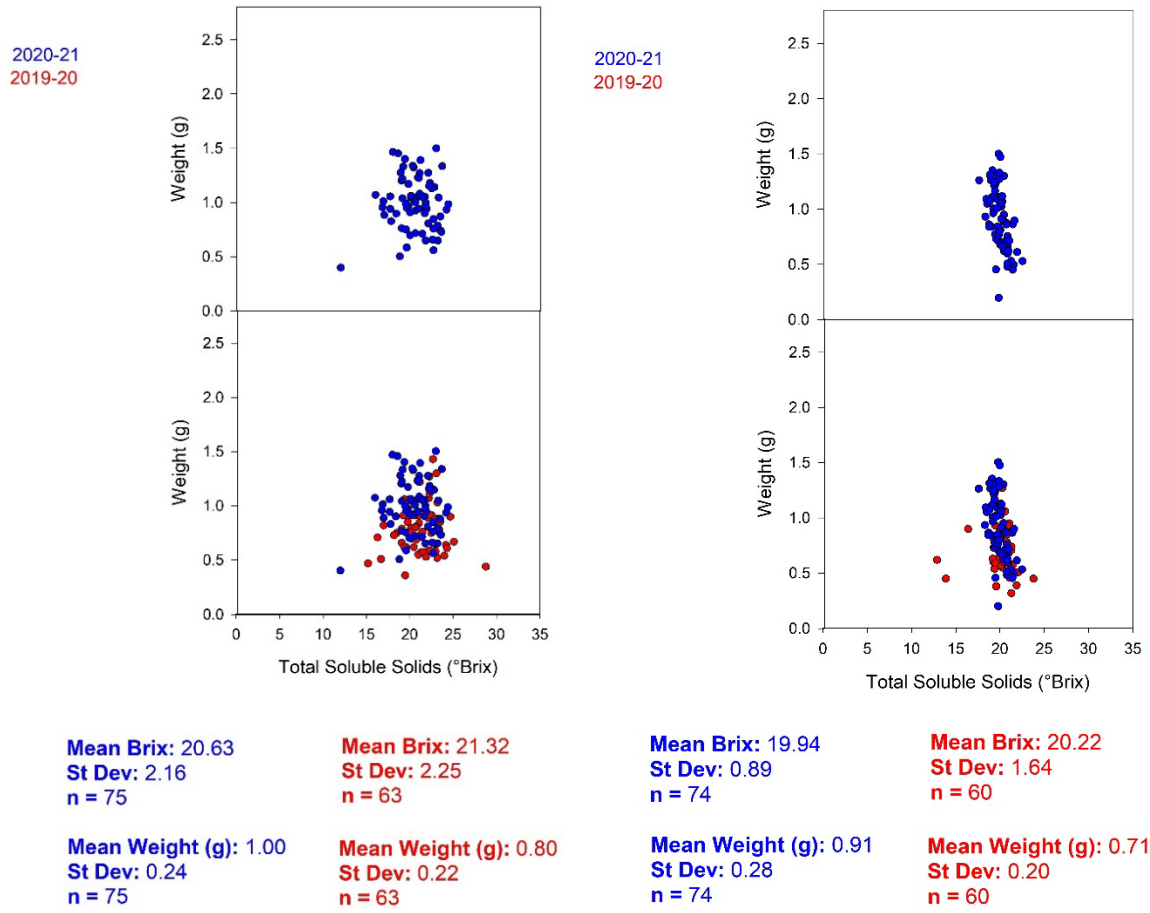


Figure 92. Berry weight vs TSS (°Brix) for two lines (left: 37-07-03, right 37-07-10) with the same parents but different berry TSS distributions. The mean TSS level for all four samples was approximately 20 °Brix. The blue symbols and text refer to data from the 2020/21 season, the red symbols and text refer to data from the 2019/20 season. The upper panels are plots of the 2020/21 data alone, the lower panels have the 2019/20 data overlaid. St Dev = standard deviation, n = number of randomly harvested berries measured.

In both the 2019/20 and 2020/21 seasons berries were sampled at harvest and stored frozen. Micro-fermentations were carried out as per the method described earlier in this report. Analysis of the wine volatiles was carried out by us using GC-MS and the data was analysed by Emily Nicholson to be used by Dr Paul Boss's group in the search for genetic markers for important wine volatiles.

The concentration of amino acids in berries was also analysed by Emily Nicholson in all lines across both years using LC-MS. There were significant ranges in the concentrations of all amino acids measured. These differences might be expected to have an influence on wine composition and flavour/aroma (Robinson et al., 2014). It was notable that there was an approximately 10-fold range in proline concentration. High proline levels are often associated with stress and protection against it (Szabados and Savouré 2010, Signorelli et al. 2015). However, proline is not metabolised by yeast and so high levels of proline can be related to low YAN levels and stuck ferments especially with ferments having high sugar (leading to high ethanol) concentrations.

The concentrations of the organic acids, tartaric and malic, were analysed in all lines across both years using LC-MS and as for the amino acids there was quite a range of concentrations for both acids (2.6-fold for tartaric acid and 34-fold for malic acid). Having higher levels of acids, particularly tartaric acid because of its stability, would be an advantage under warming climatic conditions were higher temperatures result in lower acid content. Higher acid cultivars would be desirable under hotter conditions to decrease the amount of tartrate addition to ferments resulting in a cost saving.

16.3.3 Conclusions

The phenotypic data from the two years described above seem consistent between the two seasons and are accurate enough to be used in genetic mapping to locate the loci responsible for a range of the traits assayed. The initial traits that could be analysed will be those affecting the timing of developmental events from budburst through to harvest. Once identified, the sequence and function of the genes affected can be investigated to develop a method for altering the trait via gene-editing in elite cultivars.

6.17 Testing of various agents with the potential to alter budburst timing, in-field experiments

6.17.1 Introduction

There are potentially other ways to manipulate the timing of harvest in addition to manipulating the timing of the entry of grape berries into the ripening phase (veraison). One alternative is to delay budburst, a very early step in the process. Although the exact mechanism of budburst in grapes is not known, there has been extensive study of this process in grapes and a range of woody perennials. Late winter pruning has been used to delay budburst (e.g. Friend *et al.,* 2007), as has the application of oils and alginates, which may act by restricting oxygen levels inside the bud. A number of studies have applied a range of active reagents, such as PGRs in attempts to find a means of delaying budburst, these include ABA, Ethrel, cytokinins, NAA and GA_3 (Hellman *et al.,* 2006; Zhang *et al.,* 2011; Meitha *et al.,* 2018). Scavengers of hydroxyl radicals (e.g. mannitol) and hydrogen peroxide (e.g. pyruvate) may also be useful in delaying budburst by reducing the concentration of reactive oxygen species formed as a consequence of the hypoxic state which occurs during budburst. Despite numerous reports, the effectiveness of these treatments is still not clear. Experiments in the glasshouse may not be successfully translated to the field and the results from different studies are not always consistent. We selected a range of active compounds to further test their role and effectiveness in delaying budburst. Delaying budburst rather than delaying the entry of berries into the ripening phase and hence delaying harvest, does have the advantage of not directly treating berries and the application at such an early stage allows for time for the reagent used to be metabolised reducing issues with residues.

6.17.2 Materials and methods

6.17.2.1 Bud treatments

First year – 2019/20

Spur-pruned Shiraz vines located at Willunga, SA, were used for this experiment. For each treatment ten suitable spurs carrying two buds were tagged with differently coloured/patterned flagging tape. All buds were treated by spraying to runoff. Due to the likely difficulty of penetration of the applied treatments into bud tissue and no firm information of the stage at which the treatments might be most effective the treatments were applied on four dates (2, 13, 29 Aug and 2 September). On all four dates the following treatments were applied:

- Control - 0.01% (v/v) Vitiwet, 500 µL 1 N NaOH per 500 mL
- AVG - as for Control but containing 0.835g/0.5 L 'Retain' (active ingredient AVG)
- ABA - as for Control but containing 100 mg/0.5 L ABA
- ABA/AVG - as for Control but containing ABA and AVG
- NAA - as for Control but containing 75 mg/0.5 L NAA
- Ethrel - as for Control but containing 150 µL/0.5 L Ethrel
- GA_3 - As for Control but containing 100 mg/0.5 L GA_3.

For the last two spray treatment dates, extra buds were tagged and three additional treatments were included: mannitol - as for Control, but with 1.8 g/0.5 L (20 mM) D-mannitol; sodium pyruvate - as for Control, but with 0.55 g/0.5 L (10 mM) sodium pyruvate and 6-BA (cytokinin) - as for Control but with 75 mg/0.5 L 6-BA.

Second year – 2020/21

Spur-pruned Shiraz vines located at Willunga, SA, were used for the second bud experiment. One panel (three vines) was used per treatment, and all spurs were tagged and treated. A randomised, triplicate design was employed. The treatments were: Control - 0.01% (v/v) Vitiwet; AVG – as for Control but with 1.637 g Retain per L; Mannitol + Sodium pyruvate - as for Control, but with 3.6 g/L D-Mannitol and 1.1 g/L sodium pyruvate per L. Treatments were performed as described for the previous year. Sprays were conducted on the 3, 10, 16 and 24 August (on 24 August the first buds were seen to be at the swelling and woolly stages).

6.17.3 Results and Discussion

In the first year, a wide range of PGRs and two reactive oxygen species scavenging agents were tested. The only treatments that showed any promise were D-mannitol and sodium pyruvate; the PGR treatments appeared to have no impact (Figure 93). Although the trial was a modest and preliminary one, D-mannitol and sodium pyruvate both seemed to delay budburst as can be seen from the higher percentage of closed or swollen buds and the lower percentage of green tips/leaves. In the second year it was decided to further test this outcome by using a combination of mannitol and pyruvate and to retest the possible effects of AVG. Unfortunately, in the larger trial no significant delay was observed for any of the treatments. There could be a number of reasons for this, for example, an issue with the timing of application relative to the state of bud development may have occurred as there are no external cues that show that budburst is imminent. The trial from the first year covered a wide range of agents and was not as large a scale as the second year so there may have been a bias in the results, which was revealed when fewer agents were tested on a larger scale. It is also possible that it is difficult to deliver the various potential delaying compounds into inactive buds as they are covered by scales until budburst has commenced. The main difficulty however is that any treatments designed to delay must be delivered before changes in the buds commit the buds to coming out of dormancy and developing into shoots. As our understanding of the finer details of bud development is not well understood and as there are no obvious visual clues, it is difficult to know when to make applications. This is why multiple application times were used in our experiments, but crucial times may still have been missed. For these reasons, such treatments do not seem to be convincing options to delay budburst. If possible, an application that would delay budburst and therefore ripening would be attractive to industry. In regions where early season frosts are a problem, delaying budburst would have a protective role. The ability to manipulate buds would also mean that a lower spray volume would have to be used and the likelihood of any residues in fruit would be greatly reduced.

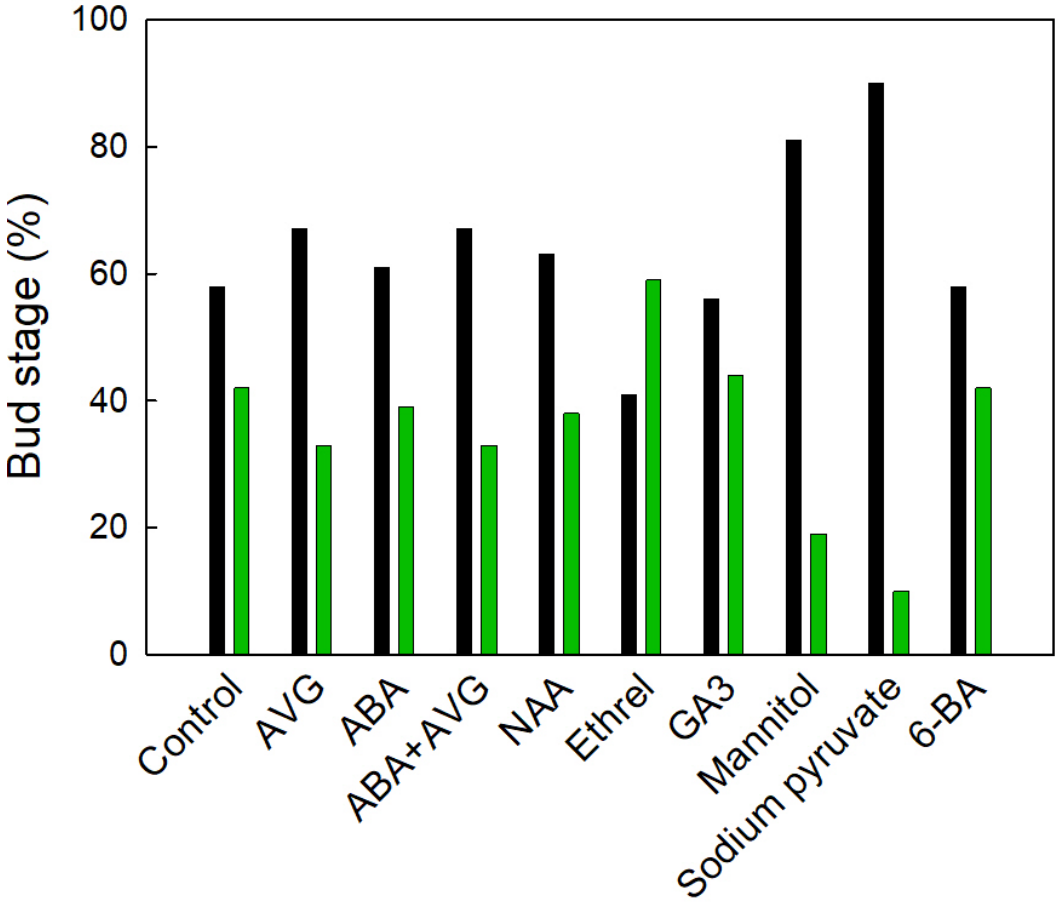


Figure 93. State of development of Shiraz buds treated with a range of PGRs, D-mannitol and sodium pyruvate. Black = closed or swollen, green = green tip or leaf

7. Outcomes and conclusions

The project was successfully completed. We believe that we have achieved, and in some cases exceeded, the targets that were set. A wide range of techniques was used to better understand the action of NAA in delaying ripening and we were fortunate enough to be able to call on a variety of expertise locally and from overseas e.g. our interaction with the University of Verona, Italy allowed us to determine the effects of NAA on the entire berry transcriptome. Of course, as there was a large field trial component to the work plan, there were the inevitable issues in some years with weather events, poor fruit set and excessively uneven fruit development, which provided challenges. Where required, these trials were repeated, sometimes in a modified format. In some cases, larger scale trials would have been appropriate, but conducting such trials in commercial vineyards can be difficult to coordinate. We believe that the data we have produced are reflective of what would occur in commercial vineyards at a larger scale.

The work presented in this report demonstrates that NAA treatment can be used to delay ripening and therefore harvest without harming the vines and fruit. We have never observed any carry-over effect into the following season as can occur with some late-pruning and shoot removal techniques used to delay harvest. The delaying treatment does not greatly alter the sensory properties of the wine made from the 'delayed' fruit. In fact, the modest changes to wine sensory character may be positive, for example, by increasing the levels of sesquiterpenes, such as rotundone, in Shiraz. The delays in harvest achieved should be long enough to assist with managing winery fruit intake and processing during what are now compressed harvest seasons due to climate change. Growers, in conjunction with winemakers, could coordinate harvest by treating some blocks to retard them in order to spread the harvest period thus improving winery processing logistics and efficiency. Being able to spread out the harvest season should also allow fruit to be harvested at the best stage for wine flavour/aroma development instead of having to harvest fruit too early, or too late, when 'green' or 'cooked' characters, respectively, may be an issue. This could improve wine value. There may also be beneficial impacts from shifting the ripening period into a cooler part of the year.

The portion of this work regarding the identification of genes that are involved in the control of agronomic and wine quality traits is forward looking. Climate change is occurring rapidly, and grapevines seem to be very sensitive to the increased temperatures. To keep pace with this change, work is needed to allow the modification of scions and rootstocks better suited to future needs. In some cases, it may be sufficient to subtly modify a single trait and for this, gene editing is the tool of choice. In other cases, more comprehensive changes may be required, particularly some related to agronomic characters.

8. Recommendations

The scientific investigation of the effects of NAA in delaying grape berry ripening is mature enough that no more of these studies should be required. The investigations have been detailed and wide-ranging. The next steps are to use this knowledge in the industry context to put the research into practice. This will require the registration of NAA for use in grapes to delay berry ripening/harvest. The initial stages of this process have commenced, and any further testing will have to be done through external providers, as is required by the APVMA. As with all agrochemicals, there will be a close scrutiny of any residue levels and the work in this study has provided important data regarding this. If this process is successful, the use of NAA would be a valuable tool to help manage harvesting timing and fruit processing, with associated economic and wine quality benefits to industry.

The studies to identify genes that control wine quality and agronomic traits, including those controlling the timing of harvest, were in the first stages at the completion of this project, but showed considerable promise. Unfortunately, this work has not been chosen for further investigation at this stage. However, this gene discovery work could be continued in the future to provide new target genes for gene editing of important traits. Having a range of targets for modification by gene editing will be a crucial part of the effort to improve planting material to adjust to a changing climate.

Appendix 1: Communication

Our work on grape berry ripening was presented to industry members through two AWRI Roadshow tours, Victoria, December 2018, Tasmania, Feb 2019. The presentation 'Understanding and manipulating grape berry development/ripening' was delivered to two different grower/winemaker audiences in each state.

The COVID pandemic has restricted other meetings/presentations but a presentation to industry entitled 'Understanding & manipulating grape berry development/ripening' was given as part of a Roadshow with AWRI, Renmark 30/11/2021.

An article "A warning for wine-lovers: climate change is messing with your favourite tipple's timing" was published on The Conversation. http://theconversation.com/a-warning-for-wine-lovers-climate-change-is-messing-with-your-favourite-tipples-timing-112865

Two media interviews regarding our work were reported:
https://www.thecitizen.org.au/articles/producing-a-decent-drop-no-doddle-as-winemakers-adapt-to-changing-climate 'Producing a decent drop no doddle as winemakers adapt to changing climate' by Jack Banister
https://www.sbs.com.au/news/climate-change-is-forcing-australia-s-struggling-winemakers-to-adapt 'Climate change is forcing Australia's struggling winemakers to adapt' Abbie O'Brian

A talk was presented at the Irrigators and Viticulture Forum, McLaren Vale, Dec 11th, 2019, 'Breakthrough research on vine stress, managing ripening and strategies for risk mitigation' Dr Chris Davies

A talk 'Understanding and manipulating small signalling molecules to affect the yield/flavour ('quality') nexus' was given in Dec 2019 as part of the recognition of our work by ADVID - Associação para o Desenvolvimento da Viticultura Duriense Edifício Centro de Excelência da Vinha e do Vinho - Régia Douro Park www.advid.pt. A diploma and an award were granted.

The following papers have been published:
Davies, C., Bottcher, C., Boss, P.K., Peat T., Newman, J. (2017) Understanding the control of grape berry ripening and developing opportunities for its manipulation. 9th International Symposium on Grapevine Physiology and Biotechnology Acta Horticulture 1157, pp.1-10 DOI 10.178660/Acta Hortic.2017.1157.1

Boss, P.K., Kalua, C.M., Nicholson, E.L., Maffei, S.M., Böttcher, C. and Davies, C. (2018) Fermentation of grapes throughout development identifies stages critical to the development of wine volatile composition. Australian Journal of Grape and Wine Research, 24, 24-37.

Böttcher, C., Boss, P.K., Harvey, K.E., Burbidge, C.A., Davies, C. (2018) Peduncle-girdling of Shiraz (Vitis vinifera L.) bunches and sugar concentration at the time of girdling affect wine volatile compounds. Australian Journal of Grape and Wine Research 24: 206-218.

Böttcher, C., Boss, P.K., Harvey, K.E., Nicholson, E.L., Burbidge, C.A., Davies, C. (2019) Post-veraison restriction of phloem import into Riesling (*Vitis vinifera* L.) berries induces transient and stable changes to fermentation-derived and varietal wine volatiles. Australian Journal of Grape and Wine Research, (DOI) - 10.1111/ajgw.12392

Dal Santo, S., Tucker, M.R., Tan, H.T., Burbidge, C.A., Fasoli, M., Böttcher, C., Boss, P.K., Pezzotti, M., Davies, C. (2020) Auxin treatment of grapevine (*Vitis vinifera* L.) berries delays ripening onset by inhibiting cell expansion. Plant Molecular Biology 103: 91-111. This paper, arising from a collaboration between CSIRO, the University of Verona (Italy) and Adelaide University, offers a mechanism to explain the action of the auxin NAA in delaying grape berry ripening.

Davies. C., Böttcher. C., Nicholson, E.L., Burbidge, C.A., Boss, P. (2021) The timing of auxin application affects the length of berry ripening/harvest delay, the synchronicity of sugar accumulation and berry growth. Australian Journal of Grape and Wine Research 28 (2), pp.232-241 doi: 10.1111/ajgw.12528

Böttcher, C., Johnson, T.E., Burbidge, C.A., Nicholson, E.L., Boss, P.K., Maffei, S.M., Bastian, E.P., Davies, C. (2021) Use of auxin to delay ripening – sensory and biochemical evaluation of Cabernet Sauvignon and Shiraz. Australian Journal of Grape and Wine Research doi: 10.1111/ajgw.12516

Dry, I.B.D., Davies, C., Dunlevy, J.D., Smith, H.M., Thomas, M.R., Walker, A.R., Walker, R.R., Clingeleffer, P.R. (2022) Development of new wine-, dried- and tablegrape scions and rootstocks for Australian viticulture: past, present and future. Australian Journal of Grape and Wine Research 28 (2), pp.177-195 doi: 10.1111/ajgw.12552

Appendix 2: Intellectual property

There is a considerable amount of data generated from the study into changes in non-volatile metabolite accumulation during the pre-veraison period. This was developed during the search for suitable marker compounds that could be used for the development of a test to accurately predict the best timing for NAA spray application for the purpose of delaying ripening. These data remain a valuable asset for the identification of metabolic markers and could also be useful for understanding the accumulation of flavour and aroma compounds and their precursors.

The berry and vine phenotypic data are extensive and valuable for the identification of traits and the respective genes that could be modified, for example by gene editing, or selected for use in a breeding program. Many of the traits measured show a great deal of variation within the populations used, which means that it should be possible to develop elite planting material with considerably improved characters. These data could be used to map the portion of the genome responsible for the desired trait, which then opens up the possibility of targeted modification through gene-editing. Gene discovery will be an important part of this process and the IP will be valuable.

Some NAA residue data are published in this report but there are more data available that would be useful IP when applying to register NAA for use in commercial vineyards to control berry ripening/harvest. Residue levels and decay profiles will be an important issue in the registration process and hence these data could have commercial value.

Appendix 3: References

Aditya J, Lewis J, Shirley NJ, Tan H-T, Henderson M, Fincher GB, Burton RA, Mather DE, Tucker MR (2015) The dynamics of cereal cyst nematode infection differ between susceptible and resistant barley cultivars and lead to changes in (1,3;1,4)-β-glucan levels and HvCslF gene transcript abundance. New Phytologist 207: 135-147

Allen MS, Lacey MJ, Boyd S (1994) Determination of methoxypyrazines in red wines by stable-isotope dilution gas-chromatography weight-spectrometry. Journal of Agriculture and Food Chemistry 42: 1734–1738

Antalick G, Šuklje K, Blackman JW, Meeks C, Deloire A, Schmidtke, LM (2015) Influence of grape composition on red wine ester profile: Comparison between Cabernet Sauvignon and Shiraz cultivars from Australian warm climate. Journal of Agricultural and Food Chemistry 63: 4664-4672

Armstrong CEJ, Ristic R, Boss PK, Pagay V, Jeffery DW (2021) Effect of grape heterogeneity on wine chemical composition and sensory attributes for Vitis vinifera cv. Cabernet Sauvignon. Australian Journal of Grape and Wine Research 27: 206-218

Ausseil A-GE, Law R, Parker AK, Teixeira EI, Sood A (2021) Projected wine grape cultivar shifts due to climate change in New Zealand. Frontiers in Plant Science 12: 618039

Barbez E, Dünser K, Gaidora A, Lendl T, Busch W (2017) Auxin steers root cell expansion via apoplastic pH regulation in Arabidopsis thaliana. Proceedings of the National Academy of Science USA 114:E4884-E4893

Barnavon L, Doco T, Terrier N, Ageorges A, Romieu C, Pellerin P (2001) Involvement of pectin methyl-esterase during the ripening of grape berries: partial cDNA isolation, transcript expression and changes in the degree of methyl-esterification of cell wall pectins. Phytochemistry 58: 693-701

Baumes R, Wirth J, Bureau, S Gunata Y, Razungles A (2002) Biogeneration of C13-norisoprenoid compounds: Experiments supportive for an apo-carotenoid pathway in grapevines. Analytica Chimica Acta 458: 3-14

Behnam B, Iuchi S, Fujita M, Fujita Y, Takasaki H, Osakabe Y, Yamaguchi-Shinozaki K, Kobayashi M, Shinozaki K (2013) Characterization of the promoter region of an Arabidopsis gene for 9-cis-epoxycarotenoid dioxygenase involved in dehydration-inducible transcription. DNA Research 20: 315-324

Bhargava A, Clabaugh I, To JP, Maxwell BB, Chiang Y-H, Schaller GE, Loraine A, Kieber JJ (2013) Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-seq in Arabidopsis. Plant Physiology 162: 272-294

Bisson LF, Karpel JE (2010) Genetics of yeast impacting wine quality. Annual Review of Food Science and Technology 1: 139-162

Blackford CL, Dennis EG, Keyzers RA, Schueuermann C, Trengrove RD, Boss PK (2018). Fermentation-guided natural products isolation of a grape berry triacylglyceride that enhances ethyl ester production. Molecules, doi:10.3390/molecules23010152.

Blake AW, McCartney L, Flint JE, Bolam DN, Boraston AB, Gilbert HJ, Knox JP (2006) Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding modules in prokaryotic enzymes. Journal of Biological Chemistry 281: 29321-29329

Boss PK, Böttcher C, Davies C (2014) Various influences of harvest date and fruit sugar content on different wine flavor and aroma compounds. American Journal of Enology and Viticulture 65: 341-353

Boss PK, Kalua CM, Nicholson EL, Maffei SM, Böttcher C, Davies C (2018) Fermentation of grapes throughout development identifies stages critical to the development of wine volatile composition. Australian Journal of Grape and Wine Research: 24: 24-37

Boss PK, Pearce A, Zhao Y, Nicholson E, Dennis E, Jeffery D (2015) Potential grape-derived contributions to volatile ester concentrations in wine. Molecules 20: 7845-7873

Böttcher C, Boss PK, Davies C (2011a) Acyl substrate preferences of an IAA-amido synthetase account for variations in grape (Vitis vinifera L.) berry ripening caused by different auxinic compounds indicating the importance of auxin conjugation in plant development. Journal of Experimental Botany 62: 4267-4280

Böttcher C, Boss PK, Davies, C (2012a) Delaying Riesling grape berry ripening with a synthetic auxin affects malic acid metabolism and sugar accumulation, and alters wine sensory characters. Functional Plant Biology 39: 745-753

Böttcher C, Boss PK, Davies C (2013a) Increase in cytokinin levels during ripening in developing Vitis vinifera cv. Shiraz berries. American Journal of Enology and Viticulture 64: 527-531

Böttcher C, Boss PK, Harvey KE, Burbidge CA, Davies, C (2017) Peduncle-girdling of Shiraz (Vitis vinifera L.) bunches and sugar concentration at the time of girdling affect wine volatile compounds. Australian Journal of Grape and Wine Research 24: 206-218

Böttcher C, Burbidge CA, Boss PK, Davies C (2013b) Interactions between ethylene and auxin are crucial to the control of grape (Vitis vinifera L.) berry ripening. BMC Plant Biology 13: 222

Böttcher C, Burbidge CA, Boss PK, Davies C (2015a) Changes in transcription of cytokinin metabolism and signalling genes in grape (Vitis vinifera L.) berries are associated with the ripening-related increase in isopentenyladenine. BMC Plant Biology 15: 223

Böttcher C, Burbidge CA, di Rienzo V, Boss PK, Davies, C (2015b) Jasmonic acid-isoleucine formation in grapevine (Vitis vinifera L.) by two enzymes with distinct transcription profiles. Journal of Integrative Plant Biology 57: 618-627

Böttcher C, Davies C (2012b) Hormonal control of grape berry development and ripening. In: Gerós H, Chaves MM, Delrot S (eds) The Biochemistry of the Grape Berry, vol 1. Bentham Science Publishers, pp 194-217

Böttcher C, Dennis EG, Booker GW, Polyak SW, Boss PK, Davies C. (2012c) A novel tool for studying auxin-metabolism: The inhibition of grapevine indole-3-acetic acid-amido synthetases by a reaction intermediate analogue. PLoS One 7, e37632

Böttcher C, Harvey KE, Boss PK, Davies C (2013b) Ripening of grape berries can be advanced or delayed by reagents that either reduce or increase ethylene levels. Functional Plant Biology 40: 566-581

Böttcher C, Harvey K, Forde CG, Boss PK, Davies C (2011b) Auxin treatment of pre-veraison grape (Vitis vinifera L.) berries both delays ripening and increases the synchronicity of sugar accumulation. Australian Journal of Grape and Wine Research 17: 1-8

Böttcher C, Keyzers RA, Boss PK, Davies C (2010) Sequestration of auxin by the indole-3-acetic acid-amido synthetase GH3-1 in grape berry (Vitis vinifera L.) and the proposed role of auxin conjugation during ripening. Journal of Experimental Botany 61: 3615-3625

Bouché N, Fromm H (2004) GABA in plants: Just a metabolite? Trends in Plant Science 9: 110-115

Bowers JE, Meredith CP (1997) The parentage of a classic wine grape, Cabernet Sauvignon. Nature Genetics 16: 84– 87

Cameron W, Petrie PR, Barlow EWR, Patrick CJ, Howell K, Fuentes S (2020) Advancement of grape maturity: comparison between contrasting cultivars and regions. Australian Journal of Grape and Wine Research 26: 53-67

Castellarin SD, Gambetta GA, Wada H, Krasnow MN, Cramer GR, Peterlunger E, Shackel KA, Matthews MA (2016) Characterization of major ripening events during softening in grape: Turgor, sugar accumulation, abscisic acid metabolism, colour development, and their relationship with growth. Journal of Experimental Botany 67: 709-722

Chen JX, Mao LC, Lu WJ, Ying TJ, Luo ZS (2016) Transcriptome profiling of postharvest strawberry fruit in response to exogenous auxin and abscisic acid. Planta 243: 183-197

Chen J-W, Bai KD, Cao K-F (2009) Inhibition of monoterpene biosynthesis accelerates oxidative stress and leads to enhancement of antioxidant defenses in leaves of rubber tree (Hevea brasiliensis). Acta Physiologiae Plantarum 31: 95-101.

Chisholm MG, Guiher LA, Vonah TM, Beaumont JL (1994) Comparison of some French-American hybrid wines with White Riesling using gas chromatography-olfactometry. American Journal of Enology and Viticulture 45: 201-212.

Chou H-C, Šuklje K, Antalick G, Schmidtke LM, Blackman JW (2018) Late-season Shiraz berry dehydration that alters composition and sensory traits of wine. Journal of Agricultural and Food Chemistry 66: 7750-7757

Cocuron J-C, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, Raikhel N, Wilkerson CG (2007) A gene from the cellulose synthase-like C family encodes a β-1,4 glucan synthase. Proceedings of the National Academy of Science USA 104: 8550-8555

Cook BI, Wolkovich EM (2016) Climate change decouples drought from early wine grape harvests in France. Nature Climate Change 6: 715-719

Coombe BG (1992) Research on development and ripening of the grape berry. American Journal of Enology and Viticulture 43: 101-110

Coombe BG, Bishop GR (1980) Development of the grape berry II: Changes in diameter and deformability during veraison. Australian Journal of Agricultural Research 31: 499-509

Coombe BG, Hale CR (1973) The hormone content of ripening grape berries and the effects of growth substance treatments. Plant Physiology 51: 629-634

Coombe BG, Iland PG (2004) Grape berry development and winegrape quality. Dry PR and Coombe BG, eds. Viticulture. (Winetitles: Adelaide, SA, Australia) pp. 210-248

Creasy GL, Lombard PB (1993) Vine water stress and peduncle girdling effects on pre- and post-veraison grape berry growth and deformability. American Journal of Enology and Viticulture 44: 193-197

Crump AM, Johnson TE, Wilkinson KL, Bastian SEP (2015) Influence of Oak Maturation Regimen on Composition, Sensory Properties, Quality, and Consumer Acceptability of Cabernet Sauvignon Wines. Journal of Agriculture and Food Chemistry 11:1593-1600

Dal Santo S, Tornielli GB, Zenoni S, Fasoli M, Farina L, Anesi A, Guzzo F, Delledonne M, Pezzotti M (2013) The plasticity of the grapevine berry transcriptome. Genome Biology 14:r54 doi:https://doi.org/10.1186/gb-2013-14-6-r54

Dal Santo S, Tucker MR, Tan HT, Burbidge CA, Fasoli M, Böttcher C, Boss PK, Pezzotti M, Davies C. (2020) Auxin treatment of grapevine (Vitis vinifera L.) berries delays ripening onset by inhibiting cell expansion. Plant Molecular Biology 103: 91-111

Danner L, Crump AM, Croker A, Gambetta JM, Johnson TE, Bastian SEP (2018) Comparison of Rate-All-That-Apply (RATA) and Descriptive Analysis (DA) for the Sensory Profiling of Wine. American Journal of Enology and Viticulture 69: 12-21

Davies C, Böttcher C (2014) Other hormonal signals during ripening. Nath P, Bouzayen M, Matto A. and Pech JC, eds. Fruit ripening: Physiology, signalling and genomics. (CABI: Wallingford, Oxfordshire, UK) pp. 202-216

Davies C, Nicholson EL, Böttcher C, Burbidge CA, Bastian SEP, Harvey KE, Huang A-C, Taylor DK, Boss PK (2015) Shiraz wines made from grape berries (Vitis vinifera) delayed in ripening by plant growth regulator treatment have elevated rotundone concentrations and “pepper” flavor and aroma. Journal of Agricultural and Food Chemistry 63: 2137-2144

De Bigault, Du Granrut A, Cacas J-L (2016) How very-long-chain fatty acids could signal stressful conditions in plants? Frontiers in Plant Science 7: 1490

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. The Plant Journal 4: 215-223

Delbarre A, Muller P, Imhoff V, Guern J (1996) Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198: 532-541

Demarty M, Morvan C, Thellier, M. (1984) Calcium and the cell wall. Plant, Cell and Environ. 7: 441-448

Deytieux-Belleau C, Vallet A, Donèche B, Geny L (2008) Pectin methylesterase and polygalacturonase in the developing grape skin. Plant Physiology and Biochemistry 46: 638-646

Dhonukshe P, Huang F, Galvan-Ampudia CS, Mähönen AP, Kleine-Vehn J, Xu J, Quint A, Prasad K, Friml J, Scheres B, Offringa R (2010) Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling. Development 137: 3245-3255

Duchêne E, Schneider C (2005) Grapevine and climatic changes: A glance at the situation in Alsace. Agronomy for Sustainable Development 25: 93-99

Dunlevy JD, Dennis EG, Soole KL, Perkins MV, Davies C, Boss PK (2013) A methyltransferase essential for the methoxypyrazine-derived flavour of wine. Plant Journal 75: 606-617

Dunlevy JD, Soole KL, Perkins MV, Dennis EG, Keyzers RA, Kalua CM, Boss PK (2010) Two O-methyltransferases involved in the biosynthesis of methoxypyrazines: grape-derived aroma compounds important to wine flavour. Plant Molecular Biolology 74: 77-89

Düring H, Alleweldt G, Koch R (1978) Studies on hormonal control of ripening in berries and grape vines. Acta Horticulturae 80: 397-405

Fasoli M, Dell'Anna R, Dal Santo S, Balestrini R, Sanson A, Pezzotti M, Monti F, Zenoni S (2016) Pectins, hemicelluloses and celluloses show specific dynamics in the internal and external surfaces of grape berry skin during ripening. Plant Cell Physiology 57: 1332-1349

Fasoli M, Richter CL, Zenoni S, Bertini E, Vitulo N, Dal Santo S, Dokoozlian N, Pezzotti M, Tornielli GB (2018) Timing and order of the molecular events marking the onset of berry ripening in grapevine. Plant Physiology 178: 1187-1206

Feng W, Kita D, Peaucelle A, Cartwright HN, Doan V, Duan Q, Liu M-C, Maman J, Steinhorst L, Schmitz-Thom I, Yvon R, Kudla J, Wu H-M, Cheung AY, Dinneny JR (2018) The FERONIA receptor kinase maintains cell-wall integrity during salt stress through Ca2+ signaling. Current Biology 28: 666-675.e665

Fernandes H, Pasternak O, Bujacz G, Bujacz A, Sikorski MM, Jaskolski M (2008) Lupinus luteus pathogenesis-related protein as a reservoir for cytokinin. Journal of Molecular Biology 378: 1040-1051

Fischer RL, Bennett AB (1991) Role of cell wall hydrolases in fruit ripening. Annual Review of Plant Physiology and Plant Molecular Biology 42: 675-703

Frankel EN (1983) Volatile lipid oxidation products. Progress in Lipid Research. 22: 1-33

Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P (2011) Evolution of cytokinin biosynthesis and degradation. Journal of Experimental Botany 62: 2431-2452

Friend AP, Trought MCT (2007) Delayed winter spur-pruning in New Zealand can alter yield components of Merlot grapevines. Australian Journal of Grape and Wine Research. 13: 157-164

Frioni T, Pirez FJ, Diti I, Ronney L, Poni S, Gatti M (2019) Post-budbreak pruning changes intra-spur phenology dynamics, vine productivity and berry ripening parameters in Vitis vinifera L. cv. 'Pinot Noir'. Scientia Horticulturae 256: 108584

Frioni T, Tombesi S, Silvestroni O, Lanari V, Bellincontro A, Sabbatini P, Gatti M, Poni S, Palliotti A (2016) Postbudburst spur pruning reduces yield and delays fruit sugar accumulation in Sangiovese in Central Italy. American Journal of Enology and Viticulture 67: 419–425

Fujiwara D, Kobayashi O, Yoshimoto H, Harashima S, Tamai Y (1999) Molecular mechanism of the multiple regulation of the Saccharomyces cerevisiae ATF1 gene encoding alcohol acetyltransferase. Yeast 15: 1183-1197

Gagne JM, Smalle J, Gingerich DJ, Walker JM, Yoo S-D, Yanagisawa S, Vierstra RD (2004) Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proceedings of the National Academy of Science USA 101: 6803-6808

Gatti M, Pirez FJ, Chiari G, Tombesi S, Palliotti A, Merli MC, Poni, S. (2016) Phenology, canopy aging and seasonal carbon balance as related to delayed winter pruning of Vitis vinifera L. cv. Sangiovese grapevines. Frontiers in Plant Science 7: 659

Gholami M, Coombe BG, Robinson SR (2004) Grapevine phloem sap analysis: 1-sucrose acids, potassium concentrations, seasonal and diurnal patterns. Acta Horticulturae 640: 143-153

Gidrol X, Lin WS, Dégousée N, Yip SF, Kush A (1994) Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. European Journal of Biochemistry 224: 21-28

Glad C, Regnard JL, Querou Y, Brun O, Morotgaudry JF (1992) Phloem sap exudates as a criterion for sink strength appreciations in Vitis vinifera L. cv. Pinot noir grapevines. Vitis 31: 131-138

Goda H, Sawa S, Asami T, Fujioka S, Shimada Y, Yoshida S (2004) Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. Plant Physiology 134: 1555-1573

Gou J-Y, Miller LM, Hou G, Yu X-H, Chen X-Y, Liu C-J (2012) Acetylesterase-mediated deacetylation of pectin impairs cell elongation, pollen germination, and plant reproduction. Plant Cell 24: 50-65

Gourieroux AM, Holzapfel BP, Scollary GR, McCully ME, Canny MJ, Rogiers SY (2016) The amino acid distribution in rachis xylem sap and phloem exudate of Vitis vinifera 'Cabernet Sauvignon' bunches. Plant Physiology and Biochemistry 105: 45-54

Grimplet J, Pimentel D, Agudelo-Romero P, Martinez-Zapater JM, Fortes AM (2017) The LATERAL ORGAN BOUNDARIES Domain gene family in grapevine: Genome-wide characterization and expression analyses during developmental processes and stress responses. Scientific Reports 7:15968

Gu S, Jacobs SD, McCarthy BS, Gohil HL (2012) Forcing vine regrowth and shifting fruit ripening in a warm region to enhance fruit quality in 'Cabernet Sauvignon' grapevine (Vitis vinifera L.). The Journal of Horticultural Science and Biotechnology 87: 287-292

Hagen G, Guilfoyle TJ (1985) Rapid induction of selective transcription by auxins. Molecular and Cellular Biology 5: 1197-1203

Hale CR, Coombe BG, Hawker JS (1970) Effects of ethylene and 2-chloroethylphosphonic acid on the ripening of grapes. Plant Physiology 45: 620-623

Hall GE, Bondada BR, Keller M (2010) Loss of rachis cell viability is associated with ripening disorders in grapes. Journal of Experimental Botany 62: 1145-1153

Hazelwood, L.A., Daran, J.-M., van Maris, A.J.A., Pronk, J.T. and Dickinson, J.R. (2008) The Ehrlich pathway for fusel alcohol production: A century of research on Saccharomyces cerevisiae metabolism. Applied and Environmental Microbiology 74: 2259-2266

He L, Meng N, Castellarin SD, Wang Y, Sun Q, Li XY, Dong ZG, Tang XP, Duan CQ, Pan QH (2021) Combined metabolite and transcriptome profiling reveals the norisoprenoid responses in grape berries to abscisic acid and synthetic auxin. International Journal of Molecular Sciences 22: 21

He L, Ren ZY, Wang Y, Fu YQ, Li Y, Meng N, Pan QH (2020) Variation of growth-to-ripening time interval induced by abscisic acid and synthetic auxin affecting transcriptome and flavor compounds in Cabernet Sauvignon grape berry. Plants-Basel 9: 19

Hellman E, Shelby S, Lowery C (2006) Exogenously applied abscisic acid did not consistently delay budburst of deacclimating grapevines. Journal of the American pomological Society 60: 178-186

Hirose N, Takei K, Kuroha T, Kamada-Nobusada T, Hayashi H, Sakakibara H (2008) Regulation of cytokinin biosynthesis, compartmentalization and translocation. Journal of Experimental Botany 59: 75-83

Hoff RT, Bondada BR, Keller M (2020) Onset and progression of the berry shrivel ripening disorder in grapes. Australian Journal of Grape and Wine Research. 2020 27: 280-289

Huang X-M, Huang H-B (2001) Early post-veraison growth in grapes: Evidence for a two-step mode of berry enlargement. Australian Journal of Grape and Wine Research 7: 132-136

Huang AC, Sumby CJ, Tiekink ERT, Taylor DK (2014) Synthesis of Guaia-4(5)-en-11-ol, Guaia-5(6)-en-11-ol, Aciphyllene, 1-epi-Melicodenones C and E, and other Guaiane-type Sesquiterpenoids via the Diastereoselective Expoxidation of Guaiol. Journal of Natural Products 77: 2522– 2536

Ishimaru M, Kobayashi S (2002) Expression of a xyloglucan endo-transglycosylase gene is closely related to grape berry softening. Plant Science 162: 621-628

Jardine KJ, Angela BJ, Holm JA, Lombardozzi DL, Negron-Juarez RI, Martin ST, Beller HR, Gimenez BO, Higuchi N, Chambers JQ (2017) Monoterpene 'thermometer' of tropical forest-atmosphere response to climate warming. Plant, Cell and Environment 40: 441-452

Jones GV, White MA, Cooper OR, Storchmann K (2005) Climate change and global wine quality. Climatic Change 73: 319-343

Kliewer WM (1968) Changes in the concentration of free amino acids in grape berries during maturation. American Journal of Enology and Viticulture 19: 166-174

Klingler JP, Batelli G, Zhu J-K (2010) ABA receptors: The START of a new paradigm in phytohormone signalling. Journal of Experimental Botany 61: 3199-3210

Kohno M, Takato H, Horiuchi H, Fujita K, Suzuki S (2012) Auxin-nonresponsive grape Aux/IAA19 is a positive regulator of plant growth. Molecular Biology Reporter 39: 911-917

Krasnow M, Matthews M, Shackel K (2008) Evidence for substantial maintenance of membrane integrity and cell viability in normally developing grape (Vitis vinifera L.) berries throughout development. Journal of Experimental Botany 59: 849-859

Krasnow MN, Matthews MA, Smith RJ, Benz J, Weber E, Shackel, KA (2010) Distinctive symptoms differentiate four common types of berry shrivel disorder in grape. California Agriculture 64: 155-159

Krasnow M, Weis N, Smith RJ, Benz MJ, Matthews M, Shackel K (2009) Inception, progression, and compositional consequences of a berry shrivel disorder. American Journal of Enology and Viticulture 60: 24-34

Křeček P, Skůpa P, Libus J, Naramoto S, Tejos R, Friml J, Zažímalová E (2009) The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biology 10: 249 doi:https://doi.org/10.1186/gb-2009-10-12-249

Lee DJ, Park JW, Lee HW, Kim J (2009) Genome-wide analysis of the auxin-responsive transcriptome downstream of iaa1 and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression. Journal of Experimental Botany 60: 3935-3957

Lehmann S, Funck D, Szabados L, Rentsch D (2010) Proline metabolism and transport in plant development. Amino Acids 39: 949-962.

Li J, Tao X, Li L, Mao L, Luo Z, Khan ZU, Ying T (2016) Comprehensive RNA-Seq Analysis on the Regulation of Tomato Ripening by Exogenous Auxin. PLOS ONE 11(5): e0156453. doi:10.1371/journal.pone.0156453

Liu Y-B, Lu S-M, Zhang J-F, Liu S, Lu Y-T (2007) A xyloglucan endotransglucosylase/hydrolase involves in growth of primary root and alters the deposition of cellulose in Arabidopsis. Planta 226: 1547-1560

Liu K, Yuan C, Feng S, Zhong S, Li H, Zhong J, Shen C, Liu J (2017) Genome-wide analysis and characterization of Aux/IAA family genes related to fruit ripening in papaya (Carica papaya L.). BMC Genomics 18: 351 doi:https://doi.org/10.1186/s12864-017-3722-6

Ljungdahl PO, Daignan-Fornier B (2012) Regulation of amino acid, nucleotide, and phosphate metabolism in Saccharomyces cerevisiae. Genetics 190: 885-929

Loveys BR, Düring H (1984) Diurnal changes in water relations and abscisic acid in field-grown Vitis vinifera cultivars. II. Abscisic acid changes under semi-arid conditions. New Phytologist 97: 37-47

Ma Q, Ding Y, Chang J, Sun X, Zhang L, Wei Q, Cheng Y, Chen L, Xu J, Deng X (2014) Comprehensive insights on how 2,4-dichlorophenoxyacetic acid retards senescence in post-harvest citrus fruits using transcriptomic and proteomic approaches. Journal of Experimental Botany 65: 61-74

Mähönen AP, Tusscher Kt, Siligato R, Smetana O, Díaz-Triviño S, Salojärvi J, Wachsman G, Prasad K, Heidstra R, Scheres B (2014) PLETHORA gradient formation mechanism separates auxin responses. Nature 515: 125-129

Maloney VJ, Samuels AL, Mansfield SD (2012) The endo-1,4-β-glucanase Korrigan exhibits functional conservation between gymnosperms and angiosperms and is required for proper cell wall formation in gymnosperms. New Phytologist 193: 1076-1087

Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Hugueney P, Frey A and Marion-Poll A (1996) Molecular identification of zeaxanthin epoxidase of Nicotiana plumbaginifolia, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of Arabidopsis thaliana. The EMBO Journal 15: 2331-2342

Marković-Housley Z, Degano M, Lamba D, von Roepenack-Lahaye E, Clemens S, Susani M, Ferreira F, Scheiner O, Breiteneder H (2003) Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. Journal of Molecular Biology 325: 123-133

Martin D, Chiang A, Lund S, Bohlmann J (2012) Biosynthesis of wine aroma: transcript profiles of hydroxymethylbutenyl diphosphate reductase, geranyl diphosphate synthase, and linalool/nerolidol synthase parallel monoterpenol glycoside accumulation in Gewürztraminer grapes. Planta 236: 919-929

Mathieu S, Terrier N, Procureur J, Bigey F, Günata Z (2005) A carotenoid cleavage dioxygenase from Vitis vinifera L.: Functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. Journal of Experimental Botany 56: 2721-2731

McClure BA, Guilfoyle T (1987) Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. Plant Molecular Biology 9: 611-623

Meitha K, Agudelo-Romero P, Signorelli S, Gibbs DJ, Considine JA, Foyer CH, Considine MJ (2018) Developmental control of hypoxia during bud burst in grapevine. Plant Cell and Environment 41: 1154-1170

Millery A, Duteurtre B, Boudaille JP, Maujean A (1986) Différentiation des trois cépages champenois à partir de l'analyse des acides aminés libres des moûts des récoltes 1983 et 1984. Revue Française Oenologie 103: 32-50

Mira de Orduña R (2010) Climate change associated effects on grape and wine quality and production. Food Research International 43: 1844-1855

Moore JP, Fangel JU, Willats WGT, Vivier MA (2014) Pectic-beta(1,4)-galactan, extensin and arabinogalactan-protein epitopes differentiate ripening stages in wine and table grape cell walls. Annals of Botany 114: 1279-1294

Moran MA, Bastian SE, Petrie PR, Sadras VO (2018) Late pruning impacts on chemical and sensory attributes of Shiraz wines. Australian Journal of Grape and Wine Research 24: 469-477

Moran MA, Petrie PR and Sadras VO (2017) Late pruning and carry-over effects on phenology, yield components and berry traits in Shiraz. Australian Journal of Grape and Wine Research 23: 378–389

Myung K, Hamilton-Kemp TR, Archbold DD (2006) Biosynthesis of trans-2-hexenal in response to wounding in strawberry fruit. Journal of Agricultural and Food Chemistry 54: 1442-1448

Niimi J, Boss PK, Jeffery D and Bastian SEP. (2017) Linking sensory properties and chemical composition of Vitis vinifera cv. Cabernet Sauvignon grape berries to wine. American Journal of Enology and Viticulture. 68: 357-367

Nunan KJ, Davies C, Robinson SP, Fincher GB (2001) Expression patterns of cell wall-modifying enzymes during grape berry development. Planta 214: 257-264

Nunan KJ, Sims IM, Bacic A, Robinson SP, Fincher GB (1998) Changes in cell wall composition during ripening of grape berries. Plant Physiology 118: 783-792

Ojeda H, Deloire A, Carbonneau A, Ageorges A, Romieu C (1999) Berry development of grapevines: Relations between the growth of berries and their DNA content indicate cell multiplication and enlargement. Vitis 38:145-150

Östin A, Kowalyczk M, Bhalerao RP, Sandberg G (1998) Metabolism of indole-3-acetic acid in Arabidopsis. Plant Physiology 118, 285-296

Palliotto A, Frioni T, Tombesi S, Sabbatini P, Guillermo Cruz-Castillo J, Lanari V, Silvestroni O, Gatti M, Poni S (2017) Double-pruning grapevines as a management tool to delay berry ripening and control yield. American Journal of Enology and Viticulture 68, 412–421

Paponov IA, Paponov M, Teale W, Menges M, Chakrabortee S, Murray JAH, Palme K (2008) Comprehensive transcriptome analysis of auxin responses in Arabidopsis. Molecular Plant 1: 321-337

Pasternak O, Bujacz GD, Fujimoto Y, Hashimoto Y, Jelen F, Otlewski J, Sikorski MM, Jaskolski M (2006) Crystal structure of Vigna radiata cytokinin-specific binding protein in complex with zeatin. The Plant Cell 18: 2622-2634

Pattison RJ, Csukasi F, Catala C (2014) Mechanisms regulating auxin action during fruit development. Physiologia Plantarum 151: 62-72

Peña A, Pardo JP, Ramírez J (1987) Early metabolic effects and mechanism of ammonium transport in yeast. Archives of Biochemistry and Biophysics 253: 431-438

Pěnčík A, Simonovik B, Petersson SV, Henyková E, Simon S, Greenham K, Zhang Y, Kowalczyk M, Estelle M, Zažímalová E, Novák O, Sandberg G, Ljung K (2013) Regulation of auxin homeostasis and gradients in Arabidopsis roots through the formation of the indole-3-acetic acid catabolite 2-oxindole-3-acetic acid. The Plant Cell 25: 3858-3870

Petrie PR, Brooke SJ, Moran MA, Sadras VO (2017) Pruning after budburst to delay and spread grape maturity. Australian Journal of Grape and Wine Research 23: 378–389

Petrie PR, Sadras VO (2008) Advancement of grape maturity in Australia between 1993-2006: putative causes, magnitude of trends and viticultural consequences. Australian Journal of Grape and Wine Research 14: 33-45

Petrie PR, Sadras VO (2016) Quantifying the advancement and compression of vintage. Australian and New Zealand Grapegrower and Winemaker 628: 40–41

Pineau B, Barbe JC, Van Leeuwen C, Dubourdieu D (2009) Examples of perceptive interactions involved in specific ”red-“ and “Black-berry” aromas in red wines. Journal of Agricultural and Food Chemistry 57: 3702-3708

Pineau B, Grose C, Beresford M, Sherman E, Raw V, Parker AK, Wohlers MW, Trought MCT (2017) Influence of grapevine canopy trimming and maturity variability within fruit population on the sensory properties of Pinot noir wine. Vitis 56: 1-10

Radauer C, Lackner P, Breiteneder H (2008) The Bet v 1 fold: An ancient, versatile scaffold for binding of large, hydrophobic ligands. BMC Evolutionary Biology 8: 286 doi:https://doi.org/10.1186/1471-2148-8-286

Ren H, Gray W M (2015) SAUR proteins as effectors of hormonal and environmental signals in plant growth. Molecular Plant 8: 1153-1164

Rienth M, Romieu C, Gregan R, Walsh C, Torregrosa L, Kelly MT (2014) Validation and application of an improved method for the rapid determination of proline in grape berries. Journal of Agricultural and Food Chemistry 62: 3384-3389

Robinson AL, Boss PK, Solomon PS, Trengove RD, Heymann H, Ebeler SE (2014) Origins of grape and wine aroma. Part 1. Chemical components and viticultural impacts. American Journal of Enology and Viticulture 65: 1-24

Rogiers SY, Coetzee ZA, Walker RR, Deloire A, Tyerman SD (2017) Potassium in the grape (Vitis vinifera L.) berry: Transport and function. Frontiers in Plant Science 8: 1629

Rogiers SY, Greer DH, Hatfield JM, Orchard BA, Keller M (2006) Mineral sinks within ripening grape berries (Vitis vinifera L). Vitis 45: 115-123

Rogiers SY, Holzapfel BP (2015) The plasticity of berry shrivelling in 'Shiraz': A vineyard survey. Vitis 54: 1-8

Roudier F, Gissot L, Beaudoin F, Haslam R, Michaelson L, Marion J, Molino D, Lima A, Bach L, Morin H, Tellier F, Palauqui J-C, Bellec Y, Renne C, Miquel M, DaCosta M, Vignard J, Rochat C, Markham JE, Moreau P, Napier J, Faure J-D (2010) Very-long-chain fatty acids are involved in polar auxin transport and developmental patterning in Arabidopsis. The Plant Cell 22: 364-375

Ryan D, Prenzler PD, Saliba AJ, Scollary GR (2008) The significance of low impact odorants in global odour perception. Trends in Food Science & Technology 19: 383-389

Ryona I, Pan BS, Sacks GL (2009) Rapid measurement of 3-alkyl-2-methoxypyrazine content of winegrapes to predict levels in resultant wines. Journal of Agriculture and Food Chemistry 57: 8250–8257

Sacks GL, Gates MJ, Ferry FX, Lavin EH, Kurtz AJ, Acree TE (2012) Sensory threshold of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and concentrations in young Riesling and non-Riesling wines. Journal of Agricultural and Food Chemistry 60: 2998-3004

Saerens SMG, Delvaux FR, Verstrepen KJ, Thevelein JM (2010) Production and biological function of volatile esters in Saccharomyces cerevisiae. Microbial Biotechnology 3: 165-177

Saerens SMG, Delvaux F, Verstrepen KJ, Van Dijck P, Thevelein JM, Delvaux FR (2008) Parameters affecting ethyl ester production by Saccharomyces cerevisiae during fermentation. Applied and Environmental Microbiology 74: 454-461

Sala C, Busto O, Guasch J, Zamora F (2005) Contents of 3-alkyl-2-methoxypyrazines in musts and wines from Vitis vinifera variety Cabernet Sauvignon: influence of irrigation and plantation density. Journal of the Science of Food and Agriculture 85: 1131–1136

Satoh R, Nakashima K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2002) ACTCAT, a novel cis-acting element for proline- and hypoosmolarity-responsive expression of the ProDH gene encoding proline dehydrogenase in Arabidopsis. Plant Physiology 130: 709-719

Schoenaers S, Balcerowicz D, Breen G, Hill K, Zdanio M, Mouille G, Holman TJ, Oh J, Wilson MH, Nikonorova N, Vu LD, De Smet I, Swarup R, De Vos WH, Pintelon I, Adriaensen D, Grierson C, Bennett MJ, Vissenberg K (2018) The auxin-regulated CrRLK1L kinase ERULUS controls cell wall composition during root hair tip growth. Current Biology 28: 722-732.e726

Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Biosynthesis of plant-derived flavor compounds. The Plant Journal 54: 712-732

Shivashankara KS, Laxman RH, Geetha GA, Roy TK, Srinivasa RNK, Patil VS (2013) Volatile aroma and antioxidant quality of ‘Shiraz’ grapes at different stages of ripening. International Journal of Fruit Science 13: 389-399

Siebert TE, Wood C, Elsey GM Pollnitz AP (2008) Determination of rotundone, the pepper aroma impact compound, in grapes and wine. Journal of Agriculture and Food Chemistry 56: 3745– 3748

Signorelli S, Dans PD, Coitiño EL, Borsani O, Monza J (2015) Connecting proline and □-aminobutyric acid in stressed plants through non-enzymatic reactions. PLoS One 10, e0115349

Skinkis PA, Bordelon BP, Wood KV (2008) Nitrogen fertilization of white Riesling grapes in Washington. Must and wine composition. American Journal of Enology and Viticulture 59: 440-445

Sosnowski MR, Ayres R, McCarthy G, Scott ES (2022) Winegrape cultivars (*Vitis vinifera*) vary in susceptibility to the grapevine trunk pathogens *Eutypa lata* and *Diplodia seriata.* Australian Journal of Grape and Wine Research 28: 166-174

Spayd SE, Wample RL, Evans RG, Stevens RG, Seymour BJ, Nagel CW (1994) Nitrogen fertilization of white Riesling grapes in Washington. Must and wine composition. American Journal of Enology and Viticulture 45: 34-42

Speirs J, Binney A, Collins M, Edwards E, Loveys B (2013) Expression of ABA synthesis and metabolism genes under different irrigation strategies and atmospheric VPDs is associated with stomatal conductance in grapevine (Vitis vinifera L. cv Cabernet Sauvignon). Journal of Experimental Botany 64: 1907-1916

Sponsel VM, Hedden P (2010) Gibberellin biosynthesis and inactivation. In: Davies PJ (ed) Plant Hormones: Biosynthesis, Signal Transduction, Action! Third edn. Springer Science, Dordrecht, pp 63-94. doi:https://doi.org/10.1007/978-1-4020-2686-7_4

Steel C, Schwarz L, Qiu Y, Schueuermann C, Blackman J, Clark A, Schmidtke L (2020). Thresholds for Botrytis bunchrot contamination of Chardonnay grapes based on the measurement of the fungalsterol, ergosterol. Australian Journal of Grape and Wine Research 26: 79-89

Stiles KA, Van Volkenbburgh E (2004) Role of K+ in leaf growth: K+ uptake is required for light-stimulated H+ efflux but not solute accumulation. Plant Cell and Environment 27: 315-335

Stines AP, Grubb J, Gockowiak H, Henschke PA, Høj PB, van Heeswijck R (2000) Proline and arginine accumulation in developing berries of Vitis vinifera L. in Australian vineyards: influence of vine cultivar, berry maturity and tissue type. Australian Journal of Grape and Wine Research 6: 150-158

Stines AP, Naylor DJ, Høj PB, van Heeswijck R (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in the levels of Δ1-pyrroline-5-carboxylate synthetase mRNA or protein. Plant Physiology 120: 923

Sugawara S, Mashiguchi K, Tanaka K, Hishiyama S, Sakai T, Hanada K, Kinoshita-Tsujimura K, Yu H, Dai X, Takebayashi Y, Takeda-Kamiya N, Kakimoto T, Kawaide H, Natsume M, Estelle M, Zhao Y, Hayashi K-i, Kamiya Y, Kasahara H (2015) Distinct characteristics of indole-3-acetic acid and phenylacetic acid, two common auxins in plants. Plant Cell Physiology 56: 1641-1654

Šuklje K, Zhang X, Antalick G, Clark AC, Deloire A, Schmidtke LM (2016) Berry shriveling significantly alters shiraz (Vitis vinifera L.) grape and wine chemical composition. Journal of Agricultural and Food Chemistry 64: 870-880

Sumby KM, Grbin PR, Jiranek V (2010) Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chemistry 121: 1-16

Sweetlove LJ, Beard KFM, Nunes-Nesi A, Fernie AR, Ratcliffe RG (2010) Not just a circle: Flux modes in the plant TCA cycle. Trends in Plant Science 15: 462-470

Symons GM, Davies C, Shavrukov Y, Dry IB, Reid JB, Thomas MR (2006) Grapes on steroids. Brassinosteroids are involved in grape berry ripening. Plant Physiology 140: 150-158

Szabados L, Savouré A (2010) Proline: A multifunctional amino acid. Trends in Plant Science 15, 89-97

Szyjewicz E, Rosner N, Kliewer WM (1984) Ethephon ((2-chloroethyl)phosphonic acid, ethrel, CEPA) in viticulture - a review. America Journal of Enology and Viticulture 35: 117-123.

Theologis A, Huynh TV, Davis RW (1985) Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. Journal of Molecular Biology 183: 53-68

Tilbrook J, Tyerman SD (2008) Cell death in grape berries: Varietal differences linked to xylem pressure and berry weight loss. Functional Plant Biology 35: 173-184

Torija MJ, Beltran G, Novo M, Poblet M, Rozès N, Mas A, Guillamón JM (2003) Effect of organic acids and nitrogen source on alcoholic fermentation: study of their buffering capacity. Journal of Agricultural and Food Chemistry 51: 916-922

Torrea D, Varela C, Ugliano M, Ancin-Azpilicueta C, Francis IL, Henschke PA (2011) Comparison of inorganic and organic nitrogen supplementation of grape juice – effect on volatile composition and aroma profile of Chardonnay wine fermented with Saccharomyces cerevisiae yeast. Food Chemistry 127: 1072-1083

Trainotti L, Tadiello A, Casadoro G (2007) The involvement of auxin in the ripening of climacteric fruits comes of age: The hormone plays a role of its own and has an intense interplay with ethylene in ripening peaches. Journal of Experimental Botany 58: 3299-3308

Trinh TTT, Yu BIN, Curran P, Liu S-Q (2012) Formation of aroma compounds during logan juice fermentation by Williopsis saturnus var. saturnus with the addition of selected amino acids. Journal of Food Processing and Preservation 36: 198-206

Turk EM, Fujioka S, Seto H, Shimada Y, Takatsuto S, Yoshida S, Denzel MA, Torres QI, Neff MM (2003) CYP72B1 inactivates brassinosteroid hormones: An intersection between photomorphogenesis and plant steroid signal transduction. Plant Physiology 133: 1643-1653

van Schie CCN, Haring MA, Schuurink RC (2007) Tomato linalool synthase is induced in trichomes by jasmonic acid. Plant Molecular Biology 64: 251-263

Varela C, Dry PR, Kutyna DR, Francis IL, Henschke PA, Curtin CD and Chambers PJ (2015) Strategies for reducing alcohol concentration in wine. Australian Journal of Grape and Wine Research 21: 670-679.

Venios X, Korkas E, Nisiotou A, Banilas G (2020) Grapevine responses to heat stress and global warming. Plants 9: 1754

Verhertbruggen Y, Yin L, Oikawa A, Scheller HV (2011) Mannan synthase activity in the CSLD family. Plant Signaling and Behavior 6:1 620-1623

Véry AA, Sentenac H (2003) Molecular mechanisms and regulation of K+ transport in higher plants. Annual Review of Plant Biology 54: 575-603

Villalobos-Gonzalez L, Pena-Neira A, Ibanez F, Pastenes C (2016) Long-term effects of abscisic acid (ABA) on the grape berry phenylpropanoid pathway: Gene expression and metabolite content. Plant Physiology and Biochemistry 105: 213-223

Villarreal NM, Martínez GA, Civello PM (2009) Influence of plant growth regulators on polygalacturonase expression in strawberry fruit. Plant Science 176: 749-757

Vuttipongchaikij S, Brocklehurst D, Steele-King C, Ashford DA, Gomez LD, McQueen-Mason SJ (2012) Arabidopsis GT34 family contains five xyloglucan α-1,6-xylosyltransferases. New Phytologist 195: 585-595

Walker RR, Blackmore DH, Clingeleffer PR, Holt H, Pearson W, Francis IL (2019) Effect of rootstock on yield, grape composition and wine sensory attributes of Shiraz grown in a moderately saline environment. Australian Journal of Grape and Wine Research 25: 414-429

Wheeler S, Loveys B, Ford C, Davies C (2009) The relationship between the expression of abscisic acid biosynthesis genes, accumulation of abscisic acid and the promotion of Vitis vinifera L. berry ripening by abscisic acid Australian Journal of Grape and Wine Research 15: 195-204

Wood C, Siebert TE, Parker M, Capone DL, Elsey GM, Pollnitz AP, Eggers M, Meier M, Vössing T, Widder S, Krammer G, Sefton MA, Herderich MJ (2008) From wine to pepper: Rotundone, an obscure sesquiterpene, is a potent spicy aroma compound. Journal of Agricultural and Food Chemistry 56: 3738-3744

Wood JA, Tan H-T, Collins HM, Yap K, Khor SF, Lim WL, Xing X, Bulone V, Burton RA, Fincher GB, Tucker MR (2018) Genetic and environmental factors contribute to variation in cell wall composition in mature desi chickpea (Cicer arietinum L.) cotyledons. Plant, Cell and Environment 41: 2195-2208

Xiong L, Zhu J-K (2003) Regulation of abscisic acid biosynthesis. Plant Physiology 133: 29-36

Xu C, Luo F, Hochholdinger F (2016) LOB domain proteins: Beyond lateral organ boundaries. Trends in Plant Science 21: 159-167

Xu YW, Lv SS, Zhao D, Chen JW, Yang WT, Wu W (2012) Effects of salicylic acid on monoterpene production and antioxidant systems in Houttuynia cordata. African Journal of Biotechnology 11: 1364-1372

Yakushiji H, Sakurai N, Morinaga K (2001) Changes in cell-wall polysaccharides from the mesocarp of grape berries during veraison. Physiologia Plantarum 111: 188-195

Young PR, Lashbrooke JG, Alexandersson E, Jacobson D, Moser C, Velasco R, Vivier MA (2012) The genes and enzymes of the carotenoid metabolic pathway in Vitis vinifera L. BMC Genomics 13: 243

Yu H, Karampelias M, Robert S, Peer WA, Swarup R, Ye S, Ge L, Cohen J, Murphy A, Friml J, Estelle M (2013) ROOT ULTRAVIOLET B-SENSITIVE1/WEAK AUXIN RESPONSE3 is essential for polar auxin transport in Arabidopsis. Plant Physiology 162:965-976

Yu P, Pickering GJ (2008) Ethanol difference thresholds in wine and the influence of mode of evaluation and wine style. American Journal of Enology and Viticulture 59: 146-152

Yuan F, Qian MC (2016) Development of C13-norisoprenoids, carotenoids and other volatile compounds in Vitis vinifera L. Cv. Pinot noir grapes. Food Chemistry 192: 633-641

Zhang Y, Mechlin T, Dami I (2011) Foliar application of abscisic acid induces dormancy responses in greenhouse-grown grapevines. Hortsceince 46: 1271-1277

Zhu B-Q, Cai J, Wang Z-Q, Xu X-Q, Duan C-Q, Pan Q-H (2014) Identification of a plastid-localized bifunctional nerolidol/linalool synthase in relation to linalool biosynthesis in young grape berries. International Journal of Molecular Sciences 15: 21992-22010

Ziliotto F, Corso M, Rizzini FM, Rasori A, Botton A, Bonghi C (2012) Grape berry ripening delay induced by a pre-véraison NAA treatment is paralleled by a shift in the expression pattern of auxin- and ethylene-related genes. BMC Plant Biol 12: 185 doi:https://doi.org/10.1186/1471-2229-12-185

Zourelidou M, Absmanner B, Weller B, Barbosa ICR, Willige BC, Fastner A, Streit V, Port SA, Colcombet J, de la Fuente van Bentem S, Hirt H, Kuster B, Schulze WX, Hammes UZ, Schwechheimer C (2014) Auxin efflux by PIN-FORMED proteins is activated by two different protein kinases, D6 PROTEIN KINASE and PINOID. eLife 3: e02860 doi:https://doi.org/10.7554/eLife.02860

Appendix 4: Staff

Christopher Davies, CSIRO Agriculture and Food, Principal Investigator

Christine Böttcher, CSIRO Agriculture and Food, Chief Investigator

Paul Boss, CSIRO Agriculture and Food, Co-investigator

Crista Burbidge, CSIRO Agriculture and Food, Technical Assistant

Sue Maffei, CSIRO Agriculture and Food, Technical Assistant

Katie Harvey, CSIRO Agriculture and Food, Technical Assistant (part time), resigned 31/1/2022

Abofazel Sarpeleh (some field work assistance and backfilling of Ms Harvey's position during 2022)

Appendix 5: Supplemental material

There is a considerable amount of detailed data arising from this project, for example, all the phenotyping measurements acquired from the CSIRO First Generation mildew resistant lines, the detailed gene expression data, that cannot possibly be recorded in its entirety in this document. These and other data will be held securely and some of the data may be used in the upcoming project involving Dr Böttcher, who is familiar with the data and its analysis.

Appendix 6: Budget reconciliation

To be provided as a separate document