EFFECT OF CLUSTER SUNLIGHT EXPOSURE ON ROTUNDONE
CONCENTRATION IN NOIRET GRAPES AND WINE

A Thesis in
Food Science
by
Laura J. Homich

© 2016 Laura J. Homich

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2016
The thesis of Laura J. Homich was reviewed and approved* by the following:

Michela Centinari  
Assistant Professor of Viticulture  
Thesis Co-Advisor

Ryan J. Elias  
Associate Professor of Food Science  
Thesis Co-Advisor

Joshua D. Lambert  
Associate Professor of Food Science

Robert Beelman  
Professor Emeritus of Food Science

Robert F. Roberts  
Professor of Food Science  
Head of the Department of Food Science

*Signatures are on file in the Graduate School.
Abstract

Laura J. Homich, Food Science, The Pennsylvania State University
Effect of Cluster Sunlight Exposure on Rotundone Concentration in Noiret Grapes and Wine

It is generally recognized that wine quality begins in the vineyard; therefore, it is important to understand how the vineyard environment and management techniques impact the development of specific compounds which contribute to or detract from perceived wine quality. The aroma impact compound rotundone was recently identified as the main contributor to the spicy, black pepper aroma in many grapes, wines, herbs, and spices. While the mechanism of rotundone formation remains unknown, it has been determined that mesoclimate plays an important role. Knowing this, studies have suggested that viticultural practices, such as leaf removal, may be a useful tool for manipulating microclimate and therefore rotundone concentrations in the vineyard and black pepper character in wines.

This two-year vineyard study sought to identify the presence of rotundone in the Noiret variety (an interspecific hybrid of Vitis) and determine if and how the timing and duration of cluster sunlight exposure affect rotundone accumulation in Noiret grapes. Another objective of this study was to understand how these sunlight exposure treatments impacted perceived black pepper aroma intensities in the vinified wines as well as
determine whether a relationship exists between the aroma intensity ratings and wine rotundone concentrations.

The timing of sunlight exposure was evaluated through comparison of pre-veraison (LR) and post-veraison (PVLR) single event leaf removal treatments, and duration was assessed through comparison of a maintained 100% sunlight exposure (MSE) treatment with an undefoliated control (CON). Enhanced point quadrat analysis (EPQA) was used to evaluate the impact of each treatment on vine canopy density and light availability in the fruiting zone. Stable isotope dilution analysis, solid phase extraction and microextraction, and gas chromatography-mass spectrometry techniques were used in combination to isolate, concentrate, and quantify rotundone in grapes. Samples collected throughout the growing season allowed for rotundone development dynamics to be monitored for each treatment. Descriptive analysis was used to evaluate the black pepper aroma intensities in wines vinified from the vineyard treatments. Furthermore, these intensity ratings were plotted against rotundone wine concentrations to determine if a correlation exists.

Many winemakers and experienced tasters have noted that the Noiret variety possesses the spicy, black pepper flavor and aroma that is associated with rotundone. Indeed, the present work confirmed this anecdotal evidence as rotundone was successfully identified for the first time in this interspecific hybrid of Vitis via gas chromatography-mass spectrometry.

Based on the findings of previous works, it was hypothesized that rotundone concentrations in Noiret grapes and wine would be reduced when viticultural practices allow for increased sunlight exposure to reach the fruiting zone during the ripening stage of development. Throughout both growing seasons, the MSE treatment reduced canopy density and increased cluster light availability compared to the CON. The timing of leaf removal treatments did not result in differences in canopy coverage or sunlight exposure in the fruiting zone at any time during 2014. In 2015, however, these treatments allowed for
increased cluster sunlight exposure following defoliation, albeit only temporarily due to vegetative re-growth in the fruiting zone. Rotundone was not detected in any of the berries sampled before veraison or at veraison. During the 2014 vintage, fruit from the CON and MSE treatments did not exhibit significant differences in rotundone concentrations at mid-ripening or harvest. In 2015, the MSE treatment yielded fruit with 45% higher rotundone concentrations than the CON at mid-ripening and 55% higher rotundone concentrations than the CON at harvest. During both seasons, rotundone concentrations in the fruit collected from the LR and PVLR vines at harvest were not significantly different. Interestingly, the PVLR treatment resulted in higher rotundone concentrations than the LR treatment in fruit sampled midway between veraison and harvest for both seasons. Vintage variation has been found to be one of the most influential factors on rotundone development with cooler vintages resulting in greater accumulations. The findings of the present study supported these previous claims as the fruit produced during the 2014 season, which was characterized by cooler temperatures, possessed significantly higher rotundone concentrations than the fruit produced during 2015. Further analysis of the relationships between fruit rotundone concentrations and vine and berry characteristics suggested that light interception during the early stages of ripening may impact the extent of rotundone formation.

With previous studies finding strong correlations between perceived pepper aroma and rotundone concentrations in wine, it was hypothesized that black pepper aroma intensity ratings would positively correlate with rotundone concentrations in the wines vinified from the viticultural treatments. Black pepper aroma intensity scores showed a positive and linear relationship ($r = 0.7906$) with rotundone concentrations in the wines.

Overall, this work successfully identified rotundone in the cold-climate interspecific hybrid Noiret. Continual leaf removal increased rotundone concentrations as compared to the undefoliated control. Post-veraison leaf removal resulted in higher rotundone concentrations in the fruit at the mid-ripening phenology stage than pre-veraison leaf
removal. Rotundone concentration varied with vintage as rotundone concentrations in the fruit were higher for the vintage that experienced less heat accumulation. Black pepper aroma intensity scores showed a positive linear relationship with rotundone concentration in wine. While further work is necessary to fully understand the mechanism responsible for the observed effects, this study suggests that rotundone concentrations may be manipulated in the vineyard through basal leaf removal.
Contents

List of Figures xi
List of Schematics xiii
List of Tables xiv
List of Abbreviations xv
Dedication xviii
Acknowledgements xix

1 Introduction 1
   1.1 Grapevine Phenology 1
   1.2 Grape Quality 3
   1.3 Fruit Zone Leaf Removal 4
   1.4 Methoxypyrazines and Leaf Removal 7
   1.5 Wine Aroma 8
      1.5.1 Grape-derived Aroma Compounds 9
      1.5.2 Formation of Aromas During Winemaking and Aging 12
      1.5.3 Aroma Interactions in the Wine Matrix 14
   1.6 Terpenes 14
   1.7 Rotundone 20
1.7.1 Discovery and Classification  
1.7.2 Identification of Rotundone in Wine Grape Cultivars  
1.7.3 Localization and Development in Grapes  
1.7.4 Environmental and Vineyard Factors that Affect Rotundone Development in the Fruit  
1.7.5 Formation Mechanisms  
1.7.6 Rotundone Extraction during Winemaking  
1.7.7 Sensory Characteristics  
1.7.8 Methodology for Rotundone Analysis  
1.7.9 In Oak Aged Spirits  

1.8 Interspecific Hybrid Grape Varieties  
1.9 The Noiret Variety  
1.10 Purpose and Significance  
1.11 Hypotheses and Objectives  

2 Materials and Methods  
2.1 Vineyard Site and Experimental Design  
2.2 Canopy Characterization  
2.3 Berry Sampling and Yield Components  
2.4 Vinification  
2.5 Berry and Wine Chemical Analysis  
2.5.1 Berry Sample Chemical Analyses  
2.5.2 Wine Chemical Analyses  
2.6 Chemicals  
2.7 Sample Preparation for Rotundone Analysis  
2.7.1 Grapes  
2.7.2 Wines  

viii
2.8 Rotundone Quantitation in Whole Berry Extracts by SPME-GC-MS 61
  2.8.1 Instrument Parameters 61
  2.8.2 Method Calibration and Repeatability 62
2.9 Rotundone Quantitation in Wines by SPME-MDGC-MS 63
  2.9.1 Instrument Parameters 63
  2.9.2 Method Calibration 64
2.10 Sensory Analysis 64
  2.10.1 Panelist Training and Discrimination Test 64
  2.10.2 Black Pepper Intensity Descriptive Analysis 65
2.11 Statistical Analysis 66

3 Results and Discussion 67
  3.1 Fruit Zone Canopy Density and Light Availability 67
  3.2 Growing Season Temperatures 72
  3.3 Harvest Yield Components and Fruit Chemistries 73
  3.4 Rotundone 76
    3.4.1 Identification in the Noiret Variety 76
    3.4.2 Concentrations in Whole Berries During Growing Season 76
    3.4.3 Wine Chemistry and Rotundone Concentration 81
    3.4.4 Correlations Between Berry Rotundone Concentrations, Vineyard Parameters, and Berry Chemistries 84
  3.5 Sensory 89
    3.5.1 Discrimination of Relative Rotundone Concentrations in Wine 89
    3.5.2 Correlation Between Black Pepper Intensity Rating and Rotundone Concentration in Noiret Wines 90
4 Conclusions and Future Work

4.1 Conclusions

4.2 Future Work

Bibliography
List of Figures

1.1 Diagram of the double sigmoid growth curve of grape berries showing the three developmental stages. From Coombe and Hale 1973. .................................................................2

1.2 Chemical structure of (-)-rotundone. From Takase et al. 2015. .............................................22

1.3 Comparison of grape berry rotundone concentrations at different phenological stages during the 2012-13 and 2014-15 growing seasons. a, b, c, d denote statistical differences (p < 0.05) among ripening stages. Adapted from Zhang et al. 2016............25

1.4 Rotundone concentrations in wines produced at five levels of ripeness in 2011 (□) and in 2012 (■). Each bar represents the mean of three replicates and error bars represent interblock variability. Different letters denote statistically different (p < 0.05) rotundone concentrations. Adapted from Geffroy et al. 2014.......................................................27

3.1 Cumulative growing degree days from 1 May to harvest in Geneva, NY during 2014 and 2015.........................................................................................................................72

3.2 Comparison of monthly growing degree days from 1 May to harvest during the 2014 and 2015 growing seasons in Geneva, NY.................................................................73

3.3 A) Chromatogram of d5-rotundone and rotundone in Noiret grapes, B) mass spectrum of rotundone, corresponding to the peak at retention time 41.554 minutes. ..............76

3.4 Impact of cluster sunlight exposure (CON vs. MSE) on rotundone concentration in (A) 2014 and (B) 2015. Impact of the timing of leaf removal (LR vs. PVLR) on rotundone concentration in (C) 2014 and (D) 2015. Berry samples were collected midway between veraison and harvest (25 or 29 days after veraison) and at harvest (57 or 55 days after veraison). Each bar represents the mean of five treatment replicates with error bars expressed as ±SE. A single asterisk (*) indicates a
significant difference at p < 0.10 and double asterisks (**) indicate a significant
different at p < 0.05.................................................................80

3.5 Relationships between fruit rotundone concentrations and berry A) pH (y = 1.62 x -
4.85, p-value = 0.0063) and B) titratable acidity (y = -0.077 x + 1.20, p-value =
0.0399). The berry samples were collected on 26 Sep 2014.................................85

3.6 Relationships between fruit rotundone concentrations at harvest (28 Oct 2014) and
EPQA metrics A) leaf layer number (y = 0.32 x + 2.34, p-value = 0.0333) and B)
percent interior clusters (y = 0.014 x + 2.37, p-value = 0.0201) recorded on 12 Sep
2014.............................................................................................86

3.7 Relationship between fruit rotundone concentrations at harvest (28 Oct 2014) and A)
yield per vine (y = -0.11 x + 4.41, p-value = 0.0128) and B) crop load (y = -0.11 x +
3.87, p-value = 0.0166). ................................................................................87

3.8 Relationships between fruit rotundone concentrations midway between veraison and
harvest (25 Sep 2015) and EPQA metrics A) percent interior clusters (y = -0.0013 x +
0.29, p-value = 0.0265) and B) cluster exposure flux availability (y = 0.21 x +
0.16, p-value = 0.0138) recorded on 11 Sep 2015.............................................88

3.9 Relationship between fruit rotundone concentrations on at harvest (21 Oct 2015 and
cluster exposure flux availability (y = 1.26 x + 1.33, p-value = 0.0462) on 11 Sep
2015.............................................................................................................88

3.10 Mean black pepper intensity ratings versus rotundone concentration for 2014 and
2015 Noiret wines. Regression equation: y = 0.090 x + 32.07, p-value = 0.0225.......90
List of Schematics

1.1 Biosynthesis of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) from dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). Adapted from Davis and Croteau 2000. ................................................................. 15

1.2 Proposed mechanism of α-guaiene biosynthesis in grapevine. Adapted from Schwab and Wust 2015. .................................................................................................................................................. 35

1.3 Proposed formation mechanism of rotundone by aerial oxidation of α-guaiene. From Huang et al. 2014. ........................................................................................................................................ 36

1.4 Proposed formation mechanism for rotundone through enzymatic conversion of α-guaiene by VvSTO2. From Takase et al. 2016. .................................................................................................................. 37
List of Tables

3.1 Effect of basal leaf removal treatments on Noiret vine canopy density and light availability in the fruiting zone during 2014. .................................................................70

3.2 Effect of basal leaf removal treatments on Noiret vine canopy density and light availability in the fruiting zone during 2015. .................................................................71

3.3 Effect of basal leaf removal on Noiret harvest yield components and berry chemistries during 2014 and 2015. ........................................................................................................75

3.4 Post-malolactic fermentation chemistry for wines vinified from the Noiret sun exposure (CON vs. MSE) and leaf removal (LR vs. PVLR) treatments. Values represent the average of two fermentation replicates per treatment. ........................................84
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AWRI</td>
<td>Australian Wine Research Institute</td>
</tr>
<tr>
<td>CEFA</td>
<td>Cluster exposure flux availability</td>
</tr>
<tr>
<td>CEL</td>
<td>Cluster exposure layer</td>
</tr>
<tr>
<td>CEM</td>
<td>Calibrated exposure mapping</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DMAPP</td>
<td>Dimethylallyl pyrophosphate</td>
</tr>
<tr>
<td>EPQA</td>
<td>Enhanced point quadrat analysis</td>
</tr>
<tr>
<td>FEP</td>
<td>Fluorinated ethylene propylene</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GC-MS/MS</td>
<td>Gas chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>GC-MS-O</td>
<td>Gas chromatograph-mass spectrometry-olfactometry</td>
</tr>
<tr>
<td>GDDs</td>
<td>Growing degree days</td>
</tr>
<tr>
<td>GPP</td>
<td>Geranyl pyrophosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IBMP</td>
<td>3-isobutyl-2-methoxypyrazine</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>LEFA</td>
<td>Leaf exposure flux availability</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LEL</td>
<td>Leaf exposure layer</td>
</tr>
<tr>
<td>LLN</td>
<td>Leaf layer number</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>LR</td>
<td>Pre-veraison leaf removal</td>
</tr>
<tr>
<td>MDGC-MS</td>
<td>Multidimensional gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MEP</td>
<td>Methyl D-erythritol 4-phosphate</td>
</tr>
<tr>
<td>MLF</td>
<td>Malolactic fermentation</td>
</tr>
<tr>
<td>MP</td>
<td>Methoxypyrazine</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSE</td>
<td>'Maintained' 100% cluster sunlight exposure</td>
</tr>
<tr>
<td>MVA</td>
<td>Mevalonic acid</td>
</tr>
<tr>
<td>NAA</td>
<td>1-naphthaleneacetic acid</td>
</tr>
<tr>
<td>NEWA</td>
<td>Network for Environment and Weather Applications</td>
</tr>
<tr>
<td>NYSAES</td>
<td>New York State Agricultural Experiment Station</td>
</tr>
<tr>
<td>OLN</td>
<td>Occlusion layer number</td>
</tr>
<tr>
<td>OPP</td>
<td>Pyrophosphate</td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PDMS/DVB</td>
<td>Polydimethylsiloxane/divinylbenzene</td>
</tr>
<tr>
<td>PDO</td>
<td>Protected designation of origin</td>
</tr>
<tr>
<td>PG</td>
<td>Percent gaps</td>
</tr>
<tr>
<td>PGR</td>
<td>Plant growth regulator</td>
</tr>
<tr>
<td>PIC</td>
<td>Percent interior clusters</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PIL</td>
<td>Percent interior leaves</td>
</tr>
<tr>
<td>PPF</td>
<td>Percent photon flux</td>
</tr>
<tr>
<td>PQA</td>
<td>Point quadrat analysis</td>
</tr>
<tr>
<td>PVLR</td>
<td>Post-veraison leaf removal</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative standard deviation</td>
</tr>
<tr>
<td>SBSE</td>
<td>Stir bar sorptive extraction</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SIDA</td>
<td>Stable isotope dilution analysis</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>TA</td>
<td>Titratable acidity</td>
</tr>
<tr>
<td>TPS</td>
<td>Terpene synthase</td>
</tr>
<tr>
<td>TSS</td>
<td>Total soluble solids</td>
</tr>
<tr>
<td>VA</td>
<td>Volatile acidity</td>
</tr>
<tr>
<td>VvSTO</td>
<td><em>Vitis vinifera</em> sesquiterpene oxidase</td>
</tr>
<tr>
<td>VvTPS</td>
<td><em>Vitis vinifera</em> terpene synthase</td>
</tr>
<tr>
<td>YAN</td>
<td>Yeast assimilable nitrogen</td>
</tr>
</tbody>
</table>
Dedication

To Mom, Dad, and Andrew – for endless love, support, and encouragement
Acknowledgements

First and foremost, I would like to thank my research advisers Dr. Michela Centinari and Dr. Ryan Elias for allowing me to continue my studies at Penn State, designing a project to fit my interests, and providing guidance, support, and encouragement throughout the past two years. Michela and Ryan have instilled more knowledge upon me than I think they realize including planting and managing a vineyard, maintaining and running analytical instrumentation, navigating through a foreign country, and probably most importantly, achieving success without the need for perfection. I would also like to thank my additional committee members, Dr. Joshua Lambert and Dr. Robert Beelman, for providing valuable insights and posing thought-provoking questions, which contributed greatly to the success of this project.

I am also incredibly grateful for the generosity of Dr. Justine Vanden Heuvel, Steve Lerch, and members of the Vanden Heuvel lab at Cornell University – this study would not have been possible without them. Justine and co-workers not only provided the Noiret vineyard site for this work, but also implemented leaf removal treatments, took several field measurements, and assisted with the sensory trial via finances and personnel. I would also like to acknowledge Ms. Alina Stelick from Cornell University for her assistance in training panelists, organizing, and performing the sensory portion of this study.

Many thanks are due to Ms. Denise Gardner for sharing her vast knowledge and winemaking skills with me, helping me publish my first journal article, providing technical assistance at the drop of a hat, and supplying continual encouragement and wisdom. I would also like to thank members of the Australian Wine Research Institute, especially
Sheridan Barter and Tracey Siebert for generously donating the rotundone standards for this project and patiently fielding many questions.

I would like to give many thanks to Don Smith for traveling with me to New York several times to assist with vineyard data collection and harvest. I would also like to acknowledge Dr. Alyssa Bakke, Dr. Rich Marini, and Dr. Dan Sykes for taking time out of their busy schedules to provide assistance with data analysis.

I am forever grateful for the salary and tuition support for my graduate studies, which was provided by the Departments of Food Science and Plant Science. I would also like to thank the Pennsylvania Wine Marking and Research Program Board, supported by Pennsylvania grape growers and winemakers, for providing funding for this research study.

I would like to thank the past and present members of the Elias lab (Jared, Zach, Jean, Marlena, Charlene, Gal, Aynur, Drew, and Laurel), and the Centinari lab (Maria, Annie, and Marine) firstly for their friendships – I have truly loved getting to know each and every one of them. I am sincerely grateful for not only their willingness to provide laboratory technical assistance and help with sample collection, harvesting, and winemaking but also for celebrating with me in my successes and providing encouragement and wisdom on the days that were hard. I would also like to thank all of the undergraduate students who had a hand in the winemaking and chemical analysis for this study, especially Olivia, Jordan, and members of the enology extension undergraduate research study. It was such a pleasure getting to know and work with each of these students, and I hope that I was able to teach them something about enology and viticulture along the way.

I am also incredibly thankful for the many friendships that I have made with students within the department, my roommates, and members of Penn State Christian Grads. I will look back on these two years fondly, remembering all the love, laughter, and good times that we shared. Finally, I would like to thank my wonderful parents and brother, who have loved me, supported me in my pursuits, and supplied infinite encouragement and wisdom throughout my life.
Chapter 1

Introduction

1.1 Grapevine Phenology

Grapevines begin their annual growth cycle in the spring with bud break, a process which requires several days with average air temperatures above 7 to 10°C. Flowering occurs several weeks after bud break as a significant amount of time is required for the shoots to develop the leaves which supply carbohydrates to developing fruit. Fertilization then occurs two to three days after pollination. Following fertilization, the berry rapidly expands through cell division and cell expansion (Creasy and Creasy 2009). After anthesis, berries grow following a double sigmoid curve which is split into three growth stages (Figure 1.1).
Figure 1.1: Diagram of the double sigmoid growth curve of grape berries showing the three developmental stages. From Coombe and Hale 1973.

The first stage is a period of rapid growth characterized by rapid cell division and slight cell expansion (Creasy and Creasy 2009; Zhang et al. 2016). During this stage, sugar is not accumulating, but organic acids, primarily malic and tartaric acids, are developing. The second stage of development is often referred to as the lag phase as it is characterized by little growth, yet the seed is maturing and developing its hard outer coating. The length of this stage is dependent upon cultivar, vine management, and environmental factors. The transition from the second to the third stage of this process corresponds to a shift from the fruit growth stage to the ripening stage (Creasy and Creasy 2009). The ripening stage of development in grapes is the physiological period that begins at veraison and lasts until the fruit is mature (Coelho et al. 2006). The timing of veraison varies from year to year, which may in part cause yearly variation in harvest date. This time point also varies within vine and cluster, possibly due to differences in the timing of anthesis.
(Coombe 1992). During the ripening stage, the berry softens and becomes translucent, the skins of red cultivars change color, and berry size increases rapidly through cellular expansion (Coombe 1992; Creasy and Creasy 2009; Zhang et al. 2016). Grape composition also changes during this stage as acidity decreases through malic acid metabolism, sugar accumulates, pH increases, and polyphenols and varietal flavor and aroma compounds develop (Caputi et al. 2011; Coelho et al. 2006; Creasy and Creasy 2009). By weight, at harvest, grapes are characterized by 75 to 85% water, 15 to 25% sugar, 0.5 to 1.0% organic acids, and 0.25% pectin (Creasy and Creasy 2009).

Fruit maturity is often monitored by analyzing total soluble solids (TSS) content, titratable acidity (TA), pH, and color, with sugar and acidity being the most commonly used parameters. Additionally, it has been proposed that grape varietal characteristics may be better understood through analysis of phenolics, carotenoids, and volatile compounds. In particular, analysis of varietal volatile composition may allow for evaluation of aroma potential and determination of the optimum harvest time when maximum potential is exhibited so that grape and wine quality may be improved (Coelho et al. 2006). Kalua and Boss (2010) compared the volatile profiles of two grape varieties from fruit set to harvest, noting differences during the various phenological stages. The results of this study showed that the pre-veraison developmental stage could be critical in optimizing aroma potential as the greatest dissimilarities in aroma between the two varieties were found at four weeks post-flowering. It was suggested that this time period may coincide with a transition in the production of volatiles (Kalua and Boss 2010).

1.2 Grape Quality

Wine quality is driven by the ability to produce high-quality grapes with the properties of each component, pulp, skin, and seeds, playing a key role (Lohitnavy et al. 2010). The period from veraison to harvest is considered to be the most critical growth stage influencing grape quality as this is when the berries accumulate sugars, change
colors, and develop aroma compounds (Zhang et al. 2015b). Development is halted when
the grape clusters are harvested from the vine; therefore, the composition of the fruit at
the time of harvest is an important determinant of grape quality and potential wine

Assessment of fruit quality is often difficult as there are very few absolute and
objective measures (Creasy and Creasy 2009). Wine quality judgments are often based on
primary nonvolatile metabolites, namely sugar and acid, possibly due to the fact that these
categories can be easily quantified, but sole consideration of these factors is often
inadequate (Coombe 1992). Sugar content is commonly considered in quality assessments
as other parameters relating to quality are dependent on sugar changes in the berry. The
level of perceived sweetness is known to be affected by acid; thus, the sugar to acid
balance is another important measure. pH is also valuable in these judgments as it is
important for chemical and microbiological stability and affects the ionic forms of many
chemical constituents. Other factors such as physical damage and disease incidence are
easy visual measures of evaluation (Creasy and Creasy 2009). However, secondary
metabolites, including several classes of volatile compounds, are considered most influential
with regards to wine flavor and aroma (Coombe and McCarthy 1997). Traditionally, these
parameters would be evaluated through subjective sensory analysis in the field, yet more
recently, laboratory techniques, including chromatographic and spectrophotometric
methods, have allowed for quantification of quality characteristics such as color, tannin
content, and aroma profile composition (Creasy and Creasy 2009).

1.3 Fruit Zone Leaf Removal

Each grape chemical constituent uniquely responds to variations in environmental
factors, and it is, therefore, important to understand these relationships in an effort to
improve grape and wine quality (Zhuang et al. 2014). In addition to environmental factors
such as climate, soil composition, and topography, viticultural practices can influence the
development of aroma compounds that contribute to the varietal character of red and white wine grape varieties (Chapman et al. 2004; Ryona et al. 2008; Scheiner et al. 2010; Skinkis et al. 2010; Styger et al. 2011).

Light is fundamental to many vine functions as it is required for the photosynthetic production of carbohydrates in the plant (Creasy and Creasy 2009). Dense grapevine canopies can produce low-quality grapes due to the decreased quantity and quality of light, lower air circulation, and increased humidity in the fruiting zone (Zoecklein et al. 1992). Cluster shading results in grapes with reduced sugar content, total phenolics, and aromatics and increased TA, malic acid, potassium, and pH (Bledsoe et al. 1988; Percival et al. 1994; Zoecklein et al. 1992). The lower concentrations of TSS found in shaded fruit is attributed to lower berry temperatures (up to 10°C lower than non-shaded), less light availability to carbohydrate source leaves, and delays in the initiation of ripening. The higher observed pH values in these clusters results from the enhanced accumulation of nitrates and potassium (Percival et al. 1994). From a microbiological perspective, fruit produced under dense canopy conditions often has higher disease incidence due to poor ventilation and less pesticide penetration (Zoecklein et al. 1992). Therefore, this shaded fruit often produces low-quality wines that are described as thin, acidic, and lacking in color and flavor (Percival et al. 1994). Several canopy management practices can be implemented in the vineyard to optimize light interception to leaves and fruit, including cluster thinning, hedging, basal leaf removal (i.e. removal of the leaves at the base of the shoot, around the fruit), and shoot thinning (Creasy and Creasy 2009; Reynolds et al. 1996). The enhanced cluster sunlight exposure that results from these cultural practices can directly affect concentrations of sugars, acids, and secondary metabolites including phenolics (Downey et al. 2006), monoterpenes (Reynolds and Wardle 1989), norisoprenoids (Lee et al. 2007), and methoxypyrazines (Hashizume and Samuta 1999). The use of these management practices for microclimate improvement is especially
important in cool climate regions that experience cool, wet, and short growing seasons (Zhuang et al. 2014).

Leaf removal is a common viticultural practice where leaves are removed from the fruiting zone to increase sunlight exposure, therefore modifying grape quality characteristics, and improving disease control (Bavaresco et al. 2008; Scheiner et al. 2010). This practice has been shown to accelerate grape maturity, allowing for improved harvest fruit chemistry with increased TSS, varietal aromas, flavors, and color, and decreased TA, malic acid, and pH (Arnold and Bledsoe 1990; Creasy and Creasy 2000; Scheiner et al. 2010). Additionally, photosynthetic photon flux density, cluster temperature, and evaporation in the fruiting zone are enhanced; studies have shown that sunlight exposure can increase berry surface temperatures by as much as 13°C (Arnold and Bledsoe 1990; Geffroy et al. 2014). Increases in TSS are hypothesized to result from advanced ripening and decreased water content in exposed berries. While some have reported sugar reductions with leaf removal, it is thought that perhaps not enough leaf area was maintained to allow for the fruit to mature (Zoecklein et al. 1992). Several studies have reported berry weight reductions as a result of leaf removal, while others have observed no difference (Zoecklein et al. 1992). Reductions in TA are attributed to accelerated degradation of malic acid through enhanced malate enzyme activity when berry temperatures reach above 30°C after veraison; it has been reported that elevated temperatures do not affect tartaric or citric acid concentrations (Percival et al. 1994; Zhuang et al. 2014). Furthermore, this cultural practice has been shown to reduce potassium uptake, and these lower potassium levels were correlated with lower concentrations of organic acids (Zhuang et al. 2014). Leaf removal has been shown to enhance the development of desirable flavor and aroma compounds in the fruit (Lohitnavy et al. 2010). Sensory evaluations have supported this claim as leaf removal was found to improve varietal aromas and fruit character, reduce vegetative notes, and improve overall wine quality ratings (Lohitnavy et al. 2010; Macaulay and Morris 1993). Beyond the
impact of this practice on chemical factors, the reduction of fungal infections, specifically *Botrytis*, is another key advantage (Bavaresco et al. 2008; Creasy and Creasy 2009). Fruit rot reduction is attributed to several microclimate factors which allow for greater evaporative potential in the fruiting zone (Zoecklein et al. 1992).

As is true with all canopy management practices, the effectiveness of leaf removal is dependent upon timing, severity, cultivar, and climate, amongst many other factors (Zoecklein et al. 1992). For wine grapes, sun exposure of 60% or more is considered most beneficial. One hundred percent sun exposure is most commonly employed in cool climate regions, yet this degree of leaf removal has the potential to result in sun damage to the fruit. Therefore, the extent of leaf removal is considered to be optimum when the canopy is opened up enough to increase sun exposure without the risk of harming the fruit (Creasy and Creasy 2009).

1.4 Methoxypyrazines and Leaf Removal

Fruit zone leaf removal has been found to be effective in manipulating methoxypyrazine concentrations present in the fruit at harvest. Methoxypyrazines (MPs) are a class of grape-derived aroma compounds that impart herbaceous, musty, and unripe characteristics in wine (Ryona et al. 2008). MPs are extracted directly from the grape to the wine through vinification practices. While these compounds can contribute to the varietal character and add complexity to wines, higher concentrations impart strong vegetative notes that can mask more desirable fruit aromas (Scheiner et al. 2010). The most important MP with respect to wine flavor is 3-isobutyl-2-methoxypyrazine (IBMP). IBMP is characterized by its distinctive bell pepper aroma, and its sensory detection threshold is estimated to be 15 ng/L in red wine (de Boubee et al. 2000; Ryona et al. 2008). It is difficult to remove IBMP during the winemaking process; therefore, it is important to understand how environment and vineyard management techniques are able to influence the concentrations which result in the harvested fruit (Ryona et al. 2008).
Several studies have suggested that viticultural management practices are the most effective means of altering MP concentrations in wine (Scheiner et al. 2010). During the growing season, IBMP concentration rose after fruit set, reaching a peak concentration at veraison. A significant decrease in IBMP concentration occurs prior to harvest as the fully mature fruit typically contains less than 10% of the peak concentration found at veraison (Ryona et al. 2008; Scheiner et al. 2010). Pre-veraison cluster sunlight exposure results in lower IBMP concentrations at harvest. Ryona et al. (2008) determined that this observed decrease was a result of suppressed accumulation rather than degradation, and pre-veraison sunlight exposure resulted in greater IBMP reductions than post-veraison exposure. Furthermore, Scheiner et al. (2010) investigated the effects timing and severity of leaf removal on IBMP development throughout the growing season. The information garnered from this study supported the results of Ryona et al. The effects of the cluster light exposure treatments on IBMP reduction were not evident until at least 15 days following application. The greatest overall reductions in IBMP at harvest, when compared to the control, resulted from pre-veraison leaf removal treatments employed at 10 and 40 days post-anthesis (Scheiner et al. 2010).

1.5 Wine Aroma

The aroma and flavor of wine are key factors which allow for the distinction between many different wine varieties and styles produced throughout the world (Swiegers et al. 2005). Beyond characterizing stylistic differences between wine types, aroma compounds are integral to wine quality (Jeffery et al. 2009). The bouquet of a wine is produced through complex interactions between many volatile chemical compounds (Styger et al. 2011). While these volatiles are thought to primarily be responsible for wine aroma, they also greatly contribute to flavor perception (Black et al. 2015). Approximately 1,000 chemical compounds influence the aroma and flavor profile of a wine with each contributing differently based on potency and relative concentration (Jeffery et al. 2009;
Styger et al. 2011). Due to the complexities of wine aroma, analysis of these compounds becomes complicated as a single method is not suitable for the identification and quantification of every component within the matrix (Styger et al. 2011). Early wine aroma chemistry research focused on the major components that detract from wine quality, particularly acetic acid, and winemaking conditions that may be employed to minimize the risk of oxidative spoilage. More recently, research focus has shifted to understanding the constituents that positively contribute to grape and wine quality, which was made possible through the development of chromatography and analytical sensory analysis (Ebeler and Thorngate 2009).

Aroma-active molecules in wine can arise from several sources and processes, including 1) free or bound aroma compounds derived directly from the grape; 2) acid- and enzyme-catalyzed chemical changes resulting in modification of aromatic and non-aromatic grape constituents; 3) secondary metabolites resulting from the microbial metabolism of sugar, fatty acids, nitrogenous compounds, and cinnamic acids originating in the grapes; 4) oak derived compounds that are extracted from the wood (e.g. oak cooperage, oak chips) during fermentation and storage; and 5) chemical changes via oxidation during winery operations, storage, and through packaging materials (Jeffery et al. 2009; Robinson et al. 2014; Styger et al. 2011). The major classes of aroma compounds in wines are monoterpenes, norisoprenoids, methoxypyrazines, volatile sulfur compounds, esters, and higher (fusel) alcohols (Robinson et al. 2014).

1.5.1 Grape-derived Aroma Compounds

Grape-derived volatiles are some of the most important components impacting the character and quality of finished wines (Coelho et al. 2006). In order for grape growers and winemakers to consistently be able to manage wine quality, there is a need for understanding the specific aroma compounds that impact quality, how they are formed, and the environmental and viticultural factors that influence their development (Black et
al. 2015). Grape volatile composition changes as the fruit matures, and several studies have shown that the evolution of volatiles during development is dependent upon cultivar (Kalua and Boss 2010). Grape growers often assess fruit quality by informal sensory analysis throughout the growing season and note that grape flavor primarily develops late in the ripening stages; this observed late development may be due to the presence of aroma compounds at concentrations above their human sensory threshold levels (Coombe and McCarthy 1997). While key compounds have been identified in certain varieties, it is evident that varietal character is dependent, not just on one compound, but the overall profile of the aroma compounds present (Robinson et al. 2014). There is apparent diversity in the aroma profiles of grape cultivars and these differences are most likely due to variations in the ratios of compounds within the matrix (Styger et al. 2011).

While the composition of volatile compounds within grapes is largely determined by genome, it can also be influenced by environmental and cultural factors such as climate variation, soil characteristics, and canopy management (Liu et al. 2016). The role of climate on grape composition is often difficult to understand as many factors, such as sunlight, temperature, humidity, and rainfall, all impact the overall vineyard mesoclimate. Furthermore, vintage, specifically the year-to-year climate variation, is known to greatly influence fruit composition (Robinson et al. 2014). Therefore, the varietal flavor profile of a certain cultivar can vary considerably based on growing region with some vineyard sites allowing for the production of more flavorful, higher quality fruit (Black et al. 2015).

Several classes of volatile compounds are grape-derived, including monoterpenes, C$_{15}$-norisoprenoids, aliphatics, methoxypyrazines, and volatile sulfur compounds (Robinson et al. 2014). Some of these aroma constituents may exist in the aroma-active form in grapes, and subsequently, can be directly transferred to wine without alteration; however, in many cases, these free volatiles are present at low concentrations (Coelho et al. 2006; Gardner et al. 2011; Swiegers et al. 2005; Zhang et al. 2016). Alternatively, aroma compounds in grapes are often found at higher concentrations in their nonodorous bound
forms with many existing as glycosides (Gardner et al. 2011; Hjelmeland and Ebeler 2015). The addition of the sugar moiety onto the aroma compound within the grape increases solubility, preventing diffusion, and providing a suitable form for storage (Hjelmeland and Ebeler 2015).

Monoterpenes impart floral and citrus notes in wine (Ebeler and Thorngate 2009). Much aroma research has focused on the evolution of monoterpenes in floral varieties in an effort to understand development dynamics and the relationship between their free and bound forms (Coombe and McCarthy 1997). Monoterpene development is influenced by climate and viticultural practices, with cool climates and shade conditions resulting in lower concentrations (Ebeler and Thorngate 2009).

$C_{15}$-norisoprenoids are a diverse class of aroma compounds which contribute to the varietal character of many wine grape cultivars, especially aromatic varieties (González-Barreiro et al. 2015; Robinson et al. 2014). These compounds are derived from grape carotenoids, which accumulate in the berry exocarp prior to veraison and serve as plant tissue photo protectors either through scavenging of the singlet oxygen or by quenching the triplet state of chlorophyll, which prevents the formation of singlet oxygen (Robinson et al. 2014). $C_{15}$-norisoprenoids often possess very low sensory detection thresholds and generally occur in their nonvolatile glycosidic forms, which are later released as volatiles through fermentation (Ebeler and Thorngate 2009). Increased sunlight exposure has been shown to enhance the production of both the free and glycosylated forms of norisoprenoids, and it has been suggested that light quality, rather than intensity, governs the extent of accumulation (Robinson et al. 2014).

Methoxypyrazines provide green, herbaceous aromas in wine (Scheiner et al. 2010). These aroma compounds tend to be associated with unripe grapes and are thought to develop as a means for deterring predators (Creasy and Creasy 2009). Light exposure has been shown to decrease methoxypyrazine concentrations, and it has been determined that pre-veraison light exposure is key to this suppression (Robinson et al. 2014).
Volatile sulfur compounds are often associated with the “off-odors” such as cabbage, rotten eggs, onion, garlic, and rubber that arise from simple sulfides, disulfides, and thiols. Some sulfur-containing aroma compounds, however, are able to contribute desirable aromas such as strawberry, passion fruit, and grapefruit (Ebeler and Thorngate 2009; Swiegers et al. 2005). Varietal thiols are known to exist as odorless cysteine-bound conjugates in grapes and the thiols are cleaved by yeast during alcoholic fermentation (Styger et al. 2011).

1.5.2 Formation of Aromas During Winemaking and Aging

Yeast fermentation can contribute to wine aroma development through several mechanisms including 1) biotransformation of grape components to aroma and flavor compounds; 2) enzyme production, which transforms neutral grape components to active aroma and flavor compounds; and 3) synthesis of flavor-active primary and secondary metabolites (Styger et al. 2011). Additionally, the generation of ethanol during primary fermentation increases the solubility of odorants while also suppressing their volatility (Ebeler and Thorngate 2009). Grape-derived nonvolatile glycosidically bound aroma compounds can be released or modified to their volatile forms by the action of yeast and bacteria and their metabolism during fermentation (Coelho et al. 2006; Swiegers et al. 2005). For instance, terpene glycosides are hydrolyzed during fermentation by glycosidase enzymes produced by the yeast and the acidic fermentation conditions; this enzyme action results in the production of the aroma-active terpene directly following hydrolysis (Ebeler and Thorngate 2009; Hjelmeland and Ebeler 2015; Styger et al. 2011). C_{15}-norisoprenoid glycosides, however, may produce nonvolatile products after hydrolysis which are then converted to volatile compounds through further chemical transformation (Hjelmeland and Ebeler 2015). Volatile secondary metabolites, including volatile fatty acids, esters, higher alcohols, and carbonyls are also produced through yeast and bacterial metabolism of sugars and amino acids (Robinson et al. 2014).
Esters are one of the main volatile constituents in wine, and they are formed during fermentation (Ebeler and Thorngate 2009; Etievant 1991). Ethyl esters of organic acids tend to be most abundant in wine, while ethyl esters of fatty acids and acetates are considered most important to wine aroma due to the fruity notes that are imparted (Etievant 1991). Ester production is mainly influenced by yeast metabolism, but genotype also plays an important role (Swiegers et al. 2005). This process begins with activation of an acid through combination with coenzyme A and subsequent reaction with alcohol to form the ester (Styger et al. 2011). Ester concentrations in wine have been found to be dependent upon fermentation temperature and levels of fermentable sugars (Zoecklein et al. 1999).

Higher alcohols (or fusel oils) are alcohols produced by yeast during fermentation which contain more than two carbon atoms (Zoecklein et al. 1999). At high concentrations, these higher alcohols impart the undesirable strong, pungent smell and taste of oil, but when present at lower concentrations, they can contribute fruity notes (Etievant 1991; Swiegers et al. 2005). Fusel oils arise from amino acid and carbohydrate sources within the must, and final concentrations of these alcohols in the wine depend on factors such as yeast strain, fermentation temperature, oxygen concentration, nitrogen status, and pH (Zoecklein et al. 1999).

Additionally, malolactic fermentation (MLF) has been shown to affect wine volatiles as this process enhances fruit and buttery notes and reduces green/grassy aromas (Styger et al. 2011). Aroma compounds may also interact with nonvolatile components within the wine matrix such as proteins, polysaccharides, lipids, and polyphenols; these interactions are complex as they depend on the structural properties of both the volatile and nonvolatile molecules (Ebeler and Thorngate 2009). During the aging process, wines tend to lose varietal and fermentation aromas and develop new aromas, which are characteristic to aged wines (Styger et al. 2011).
1.5.3 Aroma Interactions in the Wine Matrix

Odorant-odorant interactions within the matrix have the potential to alter the perception of the overall wine aroma. Certain aroma compounds are known to enhance others; for instance, the presence of norisoprenoids and dimethyl sulfide increases perceived fruitiness. On the other hand, aroma compounds may be able to mask others, decreasing the prominence of certain aromatic notes within the wine matrix (Ebeler and Thorngate 2009). Excessive production of ethanol results in wines with a perceived ‘hotness,’ which often masks much of the aroma and flavor profile of the wine (Swiegers et al. 2005). Furthermore, it has also been recognized that certain combinations of aroma compounds can result in the perception of a distinctly different aroma than the perceived aromas of the individual compounds (Ebeler and Thorngate 2009).

1.6 Terpenes

Terpenes and their oxygenated forms, terpenoids, play an important role in the aroma and flavor of grapes and wine (Mattivi et al. 2011b). Terpenes are hydrocarbons constructed from the repetitive joining of branched five carbon isoprenoid units (Wedler et al. 2015). Terpenes are categorized based on size and can be separated into eight different classes based on the number of isoprene units in their structure (Davis and Croteau 2000; Logan 2015). These compounds are often polycyclic and are formed through rearrangement of acyclic precursors by terpene synthase and/or cyclase enzymes (TPSs) by way of carbocation intermediates (Wedler et al. 2015). Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) serve as precursors in terpene biosynthesis (Robinson et al. 2014; Wedler et al. 2015). In grapes, these precursors may be formed by two unique pathways the cytosolic/peroxisomal mevalonic acid pathway (MVA) or the plastidial methyl-D-erythritol 4-phosphate pathway (MEP) (Robinson et al. 2014; Schwab and Wust 2015; Wedler et al. 2015).
Scheme 1.1: Biosynthesis of geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP) from dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP).

Adapted from Davis and Croteau 2000.

IPP and DMAPP can be combined to form geranyl pyrophosphate (GPP), the 10 carbon substrate for monoterpene synthesis, via GPP synthase (Scheme 1.1) (Robinson et al. 2014; Wedler et al. 2015). Terpene synthase/cyclase enzymes act by removing the pyrophosphate (OPP) unit of the acyclic terpene backbone to form a carbocation intermediate (Wedler et al. 2015). These TPSs not only catalyze the biosynthesis of terpenoids but are also attributed to the structural diversity that is found within this class of compounds (Davis and Croteau 2000). Research regarding the role of TPSs in the monoterpene biosynthesis has shown that grapes contain various TPSs which compete for interaction with the GPP present in the plastid (Schwab and Wust 2015). In fact, the *Vitis vinifera* terpene synthase family (VoTPS) has the largest number of characterized
synthases amongst all plant species (Robinson et al. 2014). Purification of native monoterpenes synthases showed that these enzymes resulted in the formation of multiple products at fixed ratios. The observed production of various compounds from one synthase is most likely due to the fact that several highly reactive carbocation intermediates are formed during the reaction, and can be stabilized by deprotonation (Davis and Croteau 2000).

Sesquiterpenes are biosynthesized from the 15 carbon farnesyl pyrophosphate (FPP), which is synthesized from one unit of DMAPP and two units of IPP (Scheme 1.1) (May et al. 2013; May and Wüst 2014; Robinson et al. 2014; Wedler et al. 2015). The roles of both the MVA and MEP pathways in the biosynthesis of these fundamental units for sesquiterpene synthesis were evaluated by injecting berries with deuterium-labeled precursors. This study found that the majority of the terpene precursors and end products were translocated from the berry mesocarp to the exocarp (May et al. 2013). With the majority of sesquiterpenes being found in the epicuticular wax layer of the berry, the synthesis occurs almost exclusively in the exocarp (Schwab and Wust 2015). Further investigation showed that both the cytosolic MVA and plastidial MEP precursors were used in the biosynthesis of sesquiterpenes (May et al. 2013; Schwab and Wust 2015). This “metabolic cross-talk” phenomenon is known to occur in grapes; the intermediates of these pathways are not strictly compartmentalized, thus allowing for exchange (Schwab and Wust 2015). It was suggested that a homogenous pool of IPP/DMAPP exists in the cytosol, and the metabolites produced through the MEP pathway are unidirectionally transported from the plastids (May et al. 2013; Schwab and Wust 2015). After removal of the OPP group from FPP, the farnesyl carbocation can participate in five different downstream pathways: 1) humulyl carbocation pathway, 2) germacrene A pathway, 3) germacrene C pathway, 4) germacrene D pathway, or 5) nerolidol diphosphate pathway to form various sesquiterpene products (Zhang et al. 2016). Sesquiterpenes exhibit even more structural diversity than monoterpenes due to the availability of the third double bond for
reaction and the longer, more flexible FPP chain (Davis and Croteau 2000; Schwab and Wust 2015). The VeTPS sesquiterpene enzymes often can form as many as 12 different products; there are, however, several TPSs that are selective enough to result in the biosynthesis of only one major product (Davis and Croteau 2000; Schwab and Wust 2015). Plant sesquiterpenes may be formed through enzymatic pathways or downstream chemical manipulations via processes such as oxidation which often result from environmental or external stimuli (Huang et al. 2014). Sesquiterpene synthase expression has been identified in both flowers and berries, yet the composition of sesquiterpenes present is dependent upon the stage of development and grape variety (Schwab and Wust 2015; Zhang et al. 2016).

Several studies have indicated that terpenes are present at higher concentrations towards harvest than in the early stages of fruit development as the free and conjugated terpene forms often accumulate during the ripening stage. In contrast to other plant species, grapes lack the specialized anatomical structure that allows for terpene storage (Caputi et al. 2011). Therefore, while terpenes can be present as free volatiles in the mesocarp, they mainly occur as glycosylated forms within the vacuoles of exocarp cells (Caputi et al. 2011; Mattivi et al. 2011b). Terpenes in plants emit airborne signals for chemical communication with other organisms serving either as attractants for pollinators and seed dispersing predators, protectants against attack by herbivores or pathogens and may play a part in plant-to-plant signaling (Black et al. 2015; Martin et al. 2003; Wedler et al. 2015). Specifically, due to the localization of sesquiterpenes in the berry exocarp, it has been suggested that these volatile compounds may be produced as antimicrobial agents for protection against disease such as Botrytis cinerea (Schwab and Wust 2015). In studies of other plant materials, sesquiterpenes have been identified as having the ability to increase bacterial permeability and susceptibility to exogenous antimicrobial compounds (Coelho et al. 2006). Furthermore, terpenes are known to develop in oleoresins produced at
wounded and infected areas of conifers as means of protection against herbivore and pathogen attack (Martin et al. 2003).

Most of the terpenes that have been identified as important contributors to grape and wine aroma are classified as monoterpenes, which often impart citrus and floral aromas (Black et al. 2015; Zhang et al. 2016). Monoterpenes are especially important in distinguishing between “aromatic” grape varieties (e.g. Muscat, Malvasia, Gewürztraminer, Riesling) and “neutral” grape varieties (e.g. Cabernet Sauvignon, Shiraz, Lemberger, and Chardonnay); this distinction is often made based on the presence of detectable levels of geraniol, linalool, and nerol (Caputi et al. 2011; Mattivi et al. 2011b; May and Wüst 2012; Robinson et al. 2014). Monoterpenes can exist in free or glycosidically bound forms, with the bound form being more prominent at full maturity (Styger et al. 2011). Free monoterpenes develop during ripening and exist in both the berry skin and pulp, exhibiting compound and variety specific localization ratios. Cultural factors significantly influence the accumulation of monoterpenes, and several studies have found that free and bound monoterpenes concentrations both increase with enhanced sunlight exposure. While these compounds can be transferred directly from the grapes to the wine during vinification, additional chemical or biochemical transformation may occur through the winemaking and aging processes (Black et al. 2015).

Monoterpenes tend to be found at higher levels in white wine grape varieties, while sesquiterpenes are mainly identified in red wine grape varieties (Drew et al. 2016). Sesquiterpene aromas have been described as woody, spicy, sweet, floral, clove, oily, musty, and fresh as well as contributing the spicy hop character in beer (Coelho et al. 2006). Interest in the role of these higher terpenoids with respect to wine aroma has increased in the last decade following the discovery of the sesquiterpene rotundone (Drew et al. 2016; Mattivi et al. 2011b; Schwab and Wust 2015). Prior to this finding, little research had focused on sesquiterpenes in the context of grapes and wine as it was thought that these compounds are less aroma-active than monoterpenes, and therefore, do not
greatly affect wine flavor (Black et al. 2015). Sesquiterpenes are found almost exclusively in the berry skin with minimal amounts detectable in the flesh at full maturity (May et al. 2013; Schwab and Wust 2015). Compounds within this terpene class are non-detectable prior to veraison and develop in grapes from veraison to harvest; it is generally thought that peak concentrations are reached at maturity and remain constant post-maturation, yet recent studies have challenged these observations (Black et al. 2015; Coelho et al. 2006). In an investigation of volatile composition of Riesling and Cabernet Sauvignon from fruit set to harvest, Kalua and Boss (2010) found that both monoterpenes and sesquiterpenes significantly decreased towards harvest. Zhang et al. (2016) studied terpene evolution in grapes, paying special attention to the pre- veraison stage of development. Interestingly, the early stage, defined by four weeks post-flowering, had the highest concentrations of total volatiles with terpenes accounting for 24%. Terpene and total volatile concentrations decreased over time with low concentrations observed at veraison, possibly indicating a halt in production or conversion to non-volatile forms. While increasing slightly, terpene concentrations remained low until a sharp increase was observed in the last two weeks before harvest; this sharp increase was attributed to the formation of sesquiterpenes. The number of sesquiterpenes observed at the early stage of development and ripening was similar, yet the composition of the sesquiterpenes at these stages was markedly different (Zhang et al. 2016).

Sesquiterpenes have been studied in the context of several other investigations relating to wine aroma. Two compounds were identified in an investigation of volatiles produced during ripening in V. vinifera cv. Nebbiolo, showing similar accumulation trends as those described previously (Ferrandino et al. 2012). Additionally, the sesquiterpenoids α-ylangene, β-bourbonene, germacrene D, and γ-cadinene were reported as grape volatiles, which are possible varietal markers in the V. vinifera cv. ‘Baga’ variety (Coelho et al. 2006). Sesquiterpene accumulation from veraison to harvest was also studied in relation to three varietal classes: 1) Muscat/floral with high monoterpenoid content (Muscat and
Gewürztraminer); 2) non-Muscat/floral with medium monoterpane content (Riesling); and 3) neutral (Lemberger, Cabernet Sauvignon, and Shiraz) (May and Wüst 2012). Whole berries were placed in a gas chromatography (GC) vial and the aromatics emitted into the gas phase were analyzed. The non-Muscat grape varieties almost exclusively emitted sesquiterpenes and monoterpenes were only detected in the gas phase of the aromatic varieties. The ability to detect sesquiterpenes in the gas phase indicates that sesquiterpenes can be released from the skins (May and Wüst 2012). Sesquiterpene emission increased markedly during ripening, specifically in the Muscat, Riesling and Gewürztraminer berries. The greatest increase in these compounds was observed across a two week period which began midway between veraison and harvest and ended two weeks prior to harvest. Bicyclic sesquiterpene forms increased with ripening and the acyclic forms decreased towards harvest. It was also recognized that the varieties could be distinguished from one another based on their sesquiterpene profiles. Additionally, the results of this study found that vintage and region impacted the sesquiterpene profiles of Muscat, Riesling, Lemberger, and Shiraz (May and Wüst 2012).

1.7 Rotundone

1.7.1 Discovery and Classification

Shiraz (V. vinifera L.) is one of the most commonly planted grape varieties across the world, and it is the leading red wine grape variety in Australia. Many wine labels fail to describe the aromatic profile of this variety without the mention of its characteristic ‘spicy’ or ‘peppery’ notes (Herderich et al. 2012). This peppery aroma is thought to be quintessentially Australian, possibly even contributing to the ‘terroir’ for certain wines, and its presence is considered integral in high-quality Australian Shiraz (Herderich et al. 2012; Zhang et al. 2015b).
Researchers at the Australian Wine Research Institute (AWRI) set out to identify the compound responsible for this unique characteristic in an effort to better understand the aroma profile of their most prominent red wine variety. At the beginning of study, scientists came to realize that very few earlier works had focused on understanding the aroma compounds that typify red varieties (i.e. what compounds in red wine make Shiraz taste like Shiraz) (Berger 2008). Preliminary analytical and sensory analyses, aiming to track down the identity of this peppery compound, did not yield any potential suspects, so Parker et al. (2007) decided to try a different approach (Jeffery et al. 2009). Grape homogenates were analyzed by both gas chromatography-mass spectrometry (GC-MS) and sensory analysis in an effort to develop a predictive model for pepper intensity. Through this work, α-ylangene was identified as a marker for pepper aroma intensity in grapes. While this compound itself is not aroma-active and may not be directly related to the spicy aroma, it was thought that further investigations may provide insight regarding cultural factors which influence the development of this peppery compound in grapes (Parker et al. 2007).

Through scientific perseverance and the use of gas chromatography mass spectrometry olfactometry (GC-MS-O), rotundone was identified in 2008 as the compound which contributes this spicy, black pepper aroma in grapes and wine (Siebert et al. 2008). The discovery of rotundone is considered to be one of the most interesting revelations in aroma chemistry due to the fact that it contributes the characteristic aroma of black pepper, the most widely used spice in the world (Caputi et al. 2011). Prior to this finding, several studies investigating the composition of black and white pepper suggested that its distinctive flavor and aroma was created by a complex matrix composed of several odorants and piperine, which elicits a heat sensation in the mouth (Wood et al. 2008b). In addition to peppercorns, grapes, and wine, the presence of rotundone was confirmed in several herbs and spices including marjoram, geranium, nut grass, rosemary, saltbush, basil, thyme, and oregano (Wood et al. 2008a).
Rotundone was first identified in 1967 in oil extracted from *Cyperus rotundus*, more commonly known as nut grass (Kapadia et al. 1967). In 1991, it was also found in agarwood (*Aquilaria agallocha*), which is used for incense in traditional Japanese ceremonies as well as Buddhist, Hindu, and Islamic religious services (Ishihara et al. 1991; Naef 2011). Healthy *Aquilaria* wood has no odor, yet when the tree becomes wounded and infected by fungi or insects, an oleoresin, containing several volatile compounds, is produced as a healing mechanism and a means to combat further fungal growth. Agarwood and its essential oil are rare, and due to high demands in the perfumery, the price of these ingredients can range between $100 to $100,000 per kilogram, depending on quality (Naef 2011). More recently, rotundone was identified in high-quality frankincense, which is also used in the perfumery and for aromatherapy. Concentrations present in these resins were near the sensory detection threshold and the exuded aroma was characterized as woody, coniferous, and incense-like with only slight hints of black pepper (Niebler et al. 2016).

![Figure 1.2: Chemical structure of (-)-rotundone. From Takase et al. 2015.](image)

Rotundone (C_{15}H_{20}O) is a sesquiterpene ketone belonging to the guaiene family (Figure 1.2) (Mattivi et al. 2011a). This compound was the first aroma-active sesquiterpene identified in grape berries, resulting in a recent increasing interest in this class of compounds with regards to grape and wine aroma (Black et al. 2015). Rotundone is a very hydrophobic molecule, containing strong hydrophobic and weak polar sites with
most of the structure being apolar (\( \text{Log } K_{ow} = 4.98 \)) (Caputi et al. 2011; Mattivi et al. 2011a).

### 1.7.2 Identification of Rotundone in Wine Grape Cultivars

Following the discovery of rotundone in grapes and wine, this peppery compound has been identified in several *Vitis vinifera* red varieties from various growing regions: Shiraz from Australia, Japan, and New Zealand (Logan 2015; Siebert et al. 2008; Takase et al. 2015); Mourvèdre and Durif from Australia (Wood et al. 2008b); Cabernet franc, Zinfandel, and Merlot from New Zealand (Logan 2015); Cagnulari, Schioppettino, and Vespionia from Italy (Mattivi 2016); Graciano and Maturana tinta from Spain (Cullere et al. 2016); and Duras, Pineau d’aunis, and Gamay from France (Geffroy et al. 2014). Additionally, the presence of rotundone has been confirmed in the white *Vitis vinifera* variety Grüner Veltliner from Austria, Italy, and Slovakia (Mattivi 2016).

### 1.7.3 Localization and Development in Grapes

During the early stages of investigation, it was recognized that information on sesquiterpene synthesis in grapes was lacking and the site(s) of rotundone accumulation in grapes were unknown. A localization study, where the exocarp and mesocarp of peppery red wine grapes were analyzed separately, showed that rotundone was primarily found in the grape exocarp (96.2\%) with a small fraction (3.8\%) present in the mesocarp (Caputi et al. 2011). These results were further supported by similar studies of Grüner Veltliner which found that the skins were rotundone rich (98.9\%) with only trace amounts identified in the pulp (Caputi et al. 2011; Mattivi et al. 2011b).

Caputi et al. (2011) found that rotundone accumulation began at the onset of veraison, specifically at 100\% color change, and development continued until harvest. Further research revealed that rotundone concentration remained low until well after veraison (ca. three to four weeks) (Herderich et al. 2012; Logan 2015). Drastic increases in
rotundone were observed late within the ripening stage with a relatively stable plateau being reached between mid-veraison and harvest (ca. six to eight weeks post-veraison) (Geffroy et al. 2014; Logan 2015). Slight decreases in rotundone concentration in the fruit were observed when fruit was left hanging on the vine after optimum picking time (Geffroy et al. 2014). This would indicate that grape phenological stage and harvest date can greatly impact the concentration of rotundone in the harvested fruit and the resulting wine (Zhang et al. 2015b). Recently, rotundone developmental trends were analyzed in Shiraz from four weeks post-flowering to full fruit maturity (Figure 1.3). Flower caps contained higher amounts of rotundone than all berry samples prior to harvest, showing similar concentrations as fully mature berries at the time of harvest. Over the entire season, rotundone exhibited a ‘U’ shaped development trend, with a gradual decrease in concentration from pre-veraison to 80% veraison followed by a gradual increase until harvest (Zhang et al. 2016).
Figure 1.3: Comparison of grape berry rotundone concentrations at different phenological stages during the 2012-13 and 2014-15 growing seasons. a, b, c, d denote statistical differences (p < 0.05) among ripening stages. Adapted from Zhang et al. 2016.

1.7.4 Environmental and Vineyard Factors that Affect Rotundone Development in the Fruit

After learning the nature of rotundone’s development, many researchers began evaluating the impact of different environmental and viticultural factors on rotundone development in the vineyard in an effort to determine possible means for manipulating its concentration in the fruit. Firstly, the presence of rotundone in specific varieties, while being absent in others planted within the same growing region, suggests a genetic basis for its biosynthesis. Furthermore, rotundone concentration in grapes has been found to be a clonal trait. Investigations of this clonal factor began after researchers anecdotally noted that two clones of Australian Shiraz within the same vineyard exhibited differences in perceived black pepper intensities. Instrumental analysis later confirmed that the more
peppery clone did, in fact, contain higher levels of rotundone (Herderich et al. 2012). Another study of two Shiraz clones in New Zealand found that one had an almost nine-fold and five-fold higher concentration of rotundone than the other during the first year and second year, respectively (Logan 2015). A study of three Grüner Veltliner clones showed different rotundone concentrations at harvest with one clone yielding four-fold higher concentrations than the other two (Caputi et al. 2011; Mattivi et al. 2011b). Additionally, four certified clones of Duras grown in the same experimental site produced wines with different rotundone concentrations (Geffroy et al. 2015b).

Like many other grape-derived aroma compounds, rotundone biosynthesis can also be controlled by non-biological environmental and cultural factors (Geffroy et al. 2014). Rotundone development is dependent on mesoclimate with cooler vintages and vineyard sites leading to greater accumulation (Caputi et al. 2011; Herderich et al. 2012; Scarlett et al. 2014). A survey of 137 commercial Australian wines of different Vitis vinifera varieties, vintages (the early 1990s to 2006), and produced from different regions found that wines with rotundone concentrations above the aroma detection threshold were produced either in a cool region or during a cool vintage (Herderich et al. 2012). A study of within-vineyard, vine, and cluster variability of rotundone concentrations in Shiraz grapes found that the largest variations were observed between the two vintages (18-fold) followed by vineyard zones (2-fold), cluster sectors (2-fold), and shading treatments (1.7-fold). Lower rotundone concentrations resulted from the growing season with higher ambient temperature and solar exposure from veraison to harvest and lower total precipitation (Zhang et al. 2015a). Similarly, a two vintage study showed that the season with warmer average temperatures and higher total rainfall resulted in lower rotundone concentrations at harvest (Logan 2015). A multiyear study on rotundone development dynamics and effects of cultural practices on its accumulation in Duras (V. vinifera L.) grown in the Gaillac region of France found that vintage was the main factor influencing rotundone development. The first year (2011) was characterized by warmer temperatures throughout
the growing season; rotundone accumulated rapidly, reaching peak concentrations around harvest and declined slightly thereafter (Figure 1.4). In the second, cooler vintage (2012), the rotundone development was gradual with a steady concentration increase from veraison to harvest. Interestingly, despite the cooler growing season, wines from the 2012 vintage had less than half the rotundone as compared to 2011, opposing previous observations (Geffroy et al. 2014).

**Figure 1.4:** Rotundone concentrations in wines produced at five levels of ripeness in 2011 (□) and in 2012 (■). Each bar represents the mean of three replicates and error bars represent interblock variability. Different letters denote statistically different (p < 0.05) rotundone concentrations. Adapted from Geffroy et al. 2014.

The tendency for high rotundone producing vineyards to be located in cool-climate regions and the considerable differences in rotundone concentrations across seasons may suggest that weather parameters, specifically temperature and possibly rain or soil water availability, are key factors influencing rotundone development (Herderich et al. 2015). Analysis of Shiraz grapes from different regions in Japan showed that berries from the cool climate Ueda region contained over twice as much rotundone as berries from the warmer Koshu region, suggesting the influence of macroclimate (Takase et al. 2015). Additionally, through vintage comparisons of a three-year study, Logan observed a three-fold higher
rotundone concentration in the third vintage, which had lower mean temperatures and fewer growing degree days (GDDs) than the two previous years (Logan 2015). Zhang et al. (2015b) compared the rotundone concentrations present in 15 vintages of wine produced from the same vineyard site with historical weather data in order to identify the environmental parameters that significantly influence rotundone development. Variations in rotundone concentration between vintages were attributed to a combined effect of fruit zone air temperature (maximum temperature, minimum temperature, degree hours above 25°C, and GDDs from veraison to harvest) and mean daily solar exposure from veraison to harvest. Each of these weather parameters showed an inverse relationship with rotundone concentration. Additionally, it was found that weather parameters such as mean January temperature (veraison), cumulative GDDs, and seasonal mean daily solar exposure did not have a significant effect on rotundone development. Degree hours above 25°C from veraison to harvest was found to be the most significant parameter explaining variation in rotundone concentrations between vintages, and therefore, could potentially serve as a predictor for rotundone concentrations at harvest (Zhang et al. 2015b).

Cluster sunlight exposure can greatly influence the development of berry aroma and flavor compounds. This increased amount of light impacts metabolite synthesis through increases in berry surface temperatures and/or enhanced UV-B radiation, which are key factors to rotundone development, especially from veraison to harvest (Barter et al. 2015; Zhang et al. 2015b). Zhang et al. (2015a) set out to understand the role of temperature, cluster sunlight exposure, and vine vigor on rotundone concentration through their study of variation within-vineyard, vine, and grape cluster. The experimental block was divided into three zones based on plant cell density (a surrogate measure for vine vigor), electrical soil conductivity, and slope. Differences observed in rotundone concentrations in the fruit at harvest between zones were a result of variations in vine vigor and natural shading. The zones with greater extent of natural shading, corresponding to less solar radiation and lower berry temperatures, led to higher concentrations of
rotundone. Additionally, the application of artificial shading treatments (i.e. fruit zone and whole vine shading treatment) did not significantly impact rotundone concentration in the fruit as compared to a non-shaded control (Zhang et al. 2015a).

Grape yields, vine vigor, indices of fruit quality, and flavor and aroma characteristics varied within-vineyard, with these differences being attributed to variations in land characteristics (Scarlett et al. 2014). Topography and geographic aspects of the vineyard led to variations in temperature and solar radiation and are thought to directly affect rotundone concentrations (Zhang et al. 2015a). Caputi et al. (2011) noted that two distinctly different mesoclimates resulted from different geographical aspects, a very steep hillside and a foothill plain, within a single planting of Vespolina (Vitis vinifera). Investigation of rotundone concentrations arising from the two locations showed that grapes grown on the foothill plain had lower accumulation rates and harvest concentrations as compared to those grown on the hillside (Caputi et al. 2011). Scarlett et al. (2014) investigated whether rotundone concentrations in Shiraz were spatially structured as well as identifying other parameters that may influence vineyard variability. Analysis of 177 grape samples taken at various points throughout the vineyard block showed great variation as rotundone concentrations ranged from 73 to 1082 ng/kg. This observed variation within the vineyard was found to be spatially structured, not random, with concentrations above the median occurring in the south and southeast section of the block and lower values were found in the northwest section of the block. Geographical aspect and the resulting variation of temperatures within the vineyard were thought to be highly influential on rotundone concentrations. Variations in vine vigor, vineyard soils, and elevation levels were observed throughout the block. Vine vigor was found to be influenced by one or more soil properties as well as slope. The observed variations in berry rotundone concentrations, however, were not solely driven by vine vigor, and it was suggested that synthesis is affected by other factors such as soil characteristics and topography (Scarlett et al. 2014). Therefore, the variation of the land underlying the vineyard is an important
variable which influences the accumulation of rotundone in grapes (Herderich et al. 2015). In an extension study of this work, Herderich et al. (2015) quantified several secondary metabolites based on classified low, medium, and high rotundone producing areas. The marker compound α-ylangene showed a similar relative concentration pattern as rotundone. The presumed precursor α-guaiene varied six-fold across the different zones with concentrations in the low and high rotundone producing regions showing significant differences. The results of this study suggested that several key volatile compounds synthesized in grapes are spatially structured, and metabolites arising from the same biosynthesis pathway do not necessarily show the same variability and developmental trends (Herderich et al. 2015). Zhang et al. (2015a) observed that while the rotundone concentrations were different by vintage, the trend of relative differences by vineyard zone was consistent across years. The zone with the northern aspect, corresponding to the highest amount of sunlight exposure, resulted in lower concentrations of rotundone compared to the other two zones (Zhang et al. 2015a). Geffroy et al. (2016) found that wines from Auvergne had significantly higher rotundone concentrations in comparison to those from Beaujolais, Loire Valley, and South West. The cool temperatures throughout the growing season and wet conditions from veraison to harvest of the Auvergne region were attributed to these differences (Geffroy et al. 2016). Additionally, a survey of commercial Shiraz wines from Australia, New Zealand, France, and the United States showed that concentrations were highest in wines from New Zealand and France, most likely due to the lower mean and maximum temperatures during the growing season as compared to Australia and the United States (Logan 2015).

Rotundone concentrations are also affected by berry positioning within the cluster. When looking at rotundone variations within-cluster, Zhang et al. (2015a) found that the sun-facing portions of the clusters had significantly higher temperatures than those facing the inside of the canopy. Rotundone accumulation was highest in the top back sector followed by the top front sector, and then the two bottom sections. The higher rotundone
concentrations observed in the top portions of clusters may be a result of greater shading from proximal leaves or canopy coverage. The cluster top portions also have a greater density of berries whereas the bottom portion is exposed to radiated heat from the soil and have less buffering capacity to temperature changes in comparison. Maturation within the cluster may also play a role in this observed variation as the bottom sector of the cluster often reaches full maturity prior to the top (Zhang et al. 2015a).

Vine water status also influences rotundone concentration in grapes, most likely due to the influence of this parameter on vine vigor (Zhang et al. 2015b). Vine water deficits may result in a decrease in vegetative growth and natural fruit shading; this would, in turn, lead to more light interception and higher berry surface temperatures, which decrease rotundone concentrations (Zhang et al. 2015a). Geffroy et al. (2015a) found that rotundone concentration was correlated to parameters that are both directly and indirectly related to water deficit, including stem water potential, berry weight, and grape malic acid content. Irrigation treatments were found to increase rotundone concentrations, suggesting that wetter pre-veraison conditions lead to greater rotundone synthesis (Geffroy et al. 2014). Post-veraison irrigation has also been found to increase leaf area production, resulting in cooler temperatures in the fruiting zone, which would promote rotundone formation (Geffroy et al. 2014).

Rotundone development has also been linked to powdery mildew (Erysiphe necator) incidence. Powdery mildew infection is known to stimulate natural defense mechanisms in Vitis vinifera varieties. Geffroy et al. (2015b) found a positive correlation between wine rotundone concentrations and powdery mildew severity at harvest. The authors suggested that the differences in rotundone concentrations observed between different clones of Duras may have been influenced by variations in the clonal susceptibility to biotic stress (Geffroy et al. 2015b).

Due to the dependence of rotundone development on vineyard meso and microclimate, many have suggested that the concentration of this aroma compound in the
fruit can be manipulated through vineyard management techniques (Caputi et al. 2011). Logan (2015) investigated the effects of several cultural practices on rotundone accumulation in Shiraz grapes grown in New Zealand. When studying effects of the timing of leaf removal on rotundone accumulation, pre-flowering and flowering leaf removal treatments did not impact rotundone concentrations at harvest as compared to the undefoliated control. Leaf removal at veraison, however, significantly decreased rotundone concentrations at harvest compared to the control. Furthermore, different intensities of fruit sunlight exposure were studied through comparisons of 100% fruit zone leaf removal, 100% fruit zone leaf removal with artificial shade covering (to differentiate between the impact of biological shade from leaves and other physiological functions), and an undefoliated control. These leaf removal treatments did not result in significant differences in rotundone concentrations at harvest. However, it is unclear whether the leaf removal treatments resulted in significant differences in canopy density and fruit shading. It was therefore questioned whether differences in the surface area and percentage of leaves between treatments were high enough to yield a difference in fruit sun exposure; it is possible that the control canopies were not dense enough to elicit noticeable differences in fruit sun exposure and berry surface temperatures as compared to the other treatments (Logan 2015). Geffroy et al. (2014) found that 100% leaf removal at veraison resulted in a significant decrease in rotundone concentration in Duras wines as compared to the undefoliated control. While this may suggest that light exposure or berry temperatures are important to rotundone development, studies investigating the relationships between these factors and sesquiterpene development have led to mixed findings and propose that temperature is more important than light (Geffroy et al. 2014).

Reducing crop load by 50% and 90% did not impact rotundone concentrations in Shiraz grapes. However, the authors hypothesized that the control vines, with no crop reduction, did not have crop levels large enough to yield a stress response within the vine (Logan 2015). Geffroy et al. (2014) found that cluster-thinning treatments resulted in
decreased yields, increased sugar concentrations, and therefore greater ethanol concentrations in the wine; however, rotundone concentrations were not significantly different from the unthinned control.

Hormones are also known to play an important role in berry ripening, and plant growth regulators (PGR) can be used to manipulate the times at which veraison and harvest occur. Davies et al. (2015) aimed to determine changes in wine volatile profiles with the application of PGRs 1-naphthaleneacetic acid (NAA) and Ethrel® to delay ripening in Shiraz. Both PGRs resulted in delayed ripening with NAA resulting in a more considerable effect. Volatile analysis showed that NAA resulted in higher concentrations of sesquiterpenes. Rotundone was not detected in control wines, was found at concentrations below the sensory detection threshold in the Ethrel® treatment wines, and concentrations were 14.5-fold higher, and above threshold levels, in the NAA treatment wines. Several possible reasons for these increased rotundone concentrations in wines from PGR treated fruit as compared to the control were proposed by the authors. Firstly, the PGR auxin hormones may have had a direct effect on rotundone metabolism; NAA is known to have greater resistance to auxin degradation, allowing for greater persistence than in the Ethrel® treatment. Alternatively, the observed results could have been due to the delay in veraison and harvest with PGR application. This delay allowed for extension of the pre-veraison stage, and the length of veraison to harvest was extended by eight days in the NAA treatment. On the other hand, it is also possible that this shift in the veraison to harvest period resulted in climatic differences during this stage of development, which allowed for more rotundone synthesis (Davies et al. 2015).

With little known about the biosynthesis and biological function of rotundone, Geffroy et al. (2014) suggested that its production could be a natural defense mechanism, possibly through the mevalonate/jasmonic acid pathway; therefore, this group studied the impact of exogenous jasmonic acid application on rotundone development. Results of the study showed that this plant hormone application did not significantly affect rotundone
concentrations in Duras, possibly due to factors which affect the efficacy of the treatment application such as concentration, timing, volume, and penetration of the canopy (Geffroy et al. 2014).

1.7.5 Formation Mechanisms

The exact mechanism of rotundone formation and development still remains unknown. It is well-recognized that rotundone is only characteristic of certain varieties, possibly suggesting that the genes for the biosynthesis of this odorant are either absent or expressed differently in some varieties (Drew et al. 2016). Much of the recent literature has been aimed at identifying the biosynthesis pathways that give rise to the formation of the \( \alpha \)-guaiene precursor and its subsequent transformation to rotundone.

Early works hypothesized that \( \alpha \)-guaiene serves as the precursor for rotundone as it is commonly found in plants, has structural similarities, and has been identified in many of the other plant materials that also contain rotundone (Drew et al. 2016; Huang et al. 2014). \( \alpha \)-Guaiene is known to form through the germacrene A pathway by means of farnesyl pyrophosphate (FPP), the substrate for sesquiterpene formation, and a TPS (Drew et al. 2016; Zhang et al. 2016). Despite the fact that 69 TPS genes have previously been identified in the Pinot Noir (\textit{V. vinifera} L.) grapevine genome, there was not a clear TPS gene candidate for the biosynthesis of \( \alpha \)-guaiene. Drew et al. (2016) found a previous work that had identified a recombinant protein, \textit{VePNSeInt}, from Pinot Noir which produced low levels (3.5%) of \( \alpha \)-guaiene (Scheme 1.2). While attempting to encode the \textit{VePNSeInt} gene using the allele \textit{VeTPS24}, researchers discovered that a sesquiterpene synthase, later named \textit{VeGuaS}, was able to produce high levels of guaiene-like sesquiterpenes, including \( \alpha \)-guaiene (44%). Closer inspection of the amino acid sequences of the \textit{VeGuaS} and \textit{VePNSeInt} proteins revealed amino acid differences at six positions. Previous studies had shown that the specific amino acid residues located near the active FPP binding site can significantly affect the metabolites that are produced. With this in
mind, molecular modeling of these proteins was used to identify the two varying amino acid residues (T414S and V530M) that were located at the active site. Site-directed mutagenesis of the VvTPS24 cDNA was used to alter these key T414S and V530M residues to assess the effect of these changes on the volatile metabolite composition. These mutations did change the resultant product profile; still, the wild type VvGuaS produced the most abundant levels of α-guaiene (Drew et al. 2016).

![Scheme 1.2: Proposed mechanism of α-guaiene biosynthesis in grapevine.](image)

Adapted from Schwab and Wust 2015.

While a few routes for the conversion of α-guaiene to rotundone have been reported, researchers continue to debate the feasibility of each mechanism within the context of the grapevine. Huang et al. (2014) found that rotundone could be formed through the aerial oxidation of α-guaiene, suggesting possible rotundone formation through air contact rather than an enzymatic mechanism (Scheme 1.3). Further studies regarding this non-enzymatic oxidation mechanism of α-guaiene revealed the formation of several downstream products in addition to rotundone (Huang et al. 2015).
Scheme 1.3: Proposed formation mechanism of rotundone by aerial oxidation of α-guaiene. From Huang et al. 2014.

Scientists at the AWRI consider it unlikely that the aerial oxidation mechanism is the route of rotundone formation in grapes (Barter et al. 2015). This claim is based on the expectation that warmer, sun exposed grapes would result in greater rotundone concentrations due to the enhancement of oxidation reactions at elevated temperatures, a trend opposite of those observed in previous work. Furthermore, it was hypothesized that rotundone may be formed via oxidase enzymes derived from fungi, like Botrytis, as these enzymes have been successfully used for the synthesis of rotundone from α-guaiene in the flavor and fragrance industry. Specifically, it was found that rotundone can be formed via non-specific oxidation of α-guaiene by microbial laccase enzymes in the presence of oxygen and chemical mediators (Schilling et al. 2013).

Cytochrome P450 (CYP) enzymes are often key contributors to the oxidative reactions that occur in plants; therefore, Takase et al. (2016) investigated possible sesquiterpene oxidase enzymes that may be responsible for the oxidation of α-guaiene in grapevines. In this study, Pinot Noir grapevine genome sequencing allowed for the identification of V. vinifera sesquiterpene oxidases VvSTO1 to VvSTO6, three of which
(VeSTO2, VeSTO4, and VeSTO6) could be isolated from Shiraz grape exocarp via polymerase chain reaction (PCR) analysis. Microsomes from yeast cells that express these CYPs were assayed in vitro with α-guaiene, and the volatile products were analyzed by GC-MS. The VeSTO2 oxidase enzyme successfully yielded rotundone either through a one-step mechanism or a two-step oxidation mechanism with (2R)-rotundol and (2S)-rotundol serving as intermediates which are readily oxidized (Scheme 1.4). VeSTO4 and VeSTO6 gave rise to several reaction products, yet rotundone and the possible rotundol intermediates were not produced. A study of VeSTO2 activity in the presence of several possible monoterpenes, sesquiterpene, and C17-norisoprenoid substrates revealed that this enzyme selectively transformed (+)-valencene to β-nootkatol and α-guaiene to rotundone. The enzyme showed high affinity (low K_m values) for these substrates, a characteristic that has been reported for other sesquiterpene oxidases. When the VeSTO2 enzyme was assayed with both the (+)-valencene and α-guaiene substrates present, fairly small interferences were observed. Furthermore, the VeSTO2 enzyme activity in the conversion of α-guaiene to rotundone was found to be enhanced at temperatures between 30 and 40°C and a pH range between 7.0 and 8.0 (Takase et al. 2016).

**Scheme 1.4:** Proposed formation mechanism for rotundone through enzymatic conversion of α-guaiene by VeSTO2 From Takase et al. 2016.
While it is well-known that rotundone primarily exists in the berry exocarp, the location of α-guaiene and this newly discovered \( V_{eo}STO2 \) enzyme within the grape berry had not yet been investigated. A localization study of Shiraz grape berries revealed that rotundone, α-guaiene, and the \( V_{eo}STO2 \) enzyme are all present at higher concentrations in the skin than the flesh (Takase et al. 2016). These findings would support the theory that sesquiterpene biosynthesis occurs exclusively in the berry exocarp. It was noted that both (+)-valencene and β-nootkatol were not detected in the skins of mature Shiraz or Merlot berries. Valencene has been previously identified as an aromatic sesquiterpene present in grapevine flowers of both red and white varieties, possibly to serve as an attractant for pollinators or protectant against pathogens and small herbivores (Lucker et al. 2004). Rotundone accumulation in grapes is most likely regulated by both \( V_{eo}STO2 \) expression and α-guaiene biosynthesis (Takase et al. 2016). Expression levels of \( V_{eo}STO2 \) were higher in the Shiraz variety as compared to Merlot, which aligns with the higher rotundone concentrations found in Shiraz. The expression of \( V_{eo}STO2 \) had a similar trend to both α-guaiene and rotundone; α-guaiene synthesis reached peak levels two weeks prior to peak concentrations of rotundone, supporting the hypothesis that rotundone accumulation begins following peak formation of the precursor (Takase et al. 2016).

Zhang et al. (2015a) suggested that rotundone may be transported to the fruit from the leaves and stems, which act as sources and storage locations for rotundone. While this storage and transport mechanism has been found to be possible in grapes, this route is not commonly attributed to the formation of grape-derived chemical constituents (Zhang et al. 2015a).

1.7.6 Rotundone Extraction during Winemaking

During the winemaking process, small amounts (<1%) of rotundone are first released into the must through skin breakage during grape crushing operations (Caputi et al. 2011; Logan 2015). Rotundone is extracted directly from the berry skins into the wine
during the early stages of fermentation via the combination of maceration and increasing ethanol concentration (Barter et al. 2015; Caputi et al. 2011; Herderich et al. 2012). The direct transfer of this compound from the grapes to the wine suggests that additional chemical or biochemical transformation is not occurring during fermentation, and therefore, the pepper character of the finished wine is mainly influenced by the rotundone concentration in the fruit at harvest (Barter et al. 2015; Herderich et al. 2012; Zhang et al. 2015b). Caputi et al. (2011) found that 9 to 10% of the rotundone originating the fruit was present in the wine at fermentation cap formation with this amount rising to 12 to 13% at the conclusion of primary fermentation. The poor extractability was attributed to its significant hydrophobicity. Pressing from the skins and filtration resulted in significant rotundone losses possibly due to rotundone binding to the removed material. Only a small amount (ca. 5 to 6%) of the rotundone in the harvested fruit was present in the final bottled wine (Caputi et al. 2011). Interestingly, the inclusion of the leaves and stems during fermentation was found to increase rotundone concentration six-fold in the finished wines, with stems having a larger influence on the black pepper character of the wine (Capone et al. 2012). Rotundone is known to be chemically stable in wine, with minimal losses after bottling and aging (Barter et al. 2015; Zhang et al. 2015b). A study investigating rotundone scalping by bottle closures found that rotundone concentrations were unchanged with natural cork and Stelvin screw cap closures, and minimal scalping (6%) was observed for synthetic corks after 39 months of storage (Herderich et al. 2012). These results would further support the hypothesis that the pepper character of a wine is likely to remain unchanged post-bottling (Herderich et al. 2012).

1.7.7 Sensory Characteristics

While most varietal aromas are created by a combination of several different chemical components, rotundone is one of the few known aroma impact compounds, having the ability to impart its characteristic aroma with a single compound (Mattivi et
When concentrated, rotundone is thought to have a strong, burnt aroma, yet upon dilution, it supplies its characteristic black pepper aroma (Wood et al. 2008b). Grape and wine research has allowed for the identification of several monoterpenes and norisoprenoids that function as aroma impact compounds, but little is known about the sensory characteristics of higher terpenoids including sesquiterpenes (Caputi et al. 2011).

From a sensory perspective, it is important to understand the relationship between perceived black pepper aroma intensity and rotundone concentration in wine. It is also important to determine the aroma detection threshold of this compound in order to assess the potential impact of this odorant on the overall flavor and aroma of red wines. Researchers at the AWRI began their investigations of this compound’s sensory properties by first examining the relationship between perceived black pepper aroma and flavor intensity and rotundone concentration in Shiraz wine (Wood et al. 2008b). This descriptive analysis study found that the black pepper intensity ratings were well correlated with the relative rotundone concentrations in grapes. A similar evaluation of ‘peppery’ red wines showed a strong linear relationship between the perceived pepper intensity and the rotundone concentrations determined through instrumental analysis. The second portion of this sensory study aimed to determine the aroma detection thresholds of rotundone in both water and red wine. Seventy-five percent of the panelists showed sensitivities to rotundone with the ability to distinguish between relative differences in rotundone concentration when spiked into water and red wine. Thresholds were determined to be 8 ng/L in water and 16 ng/L in red wine, classifying rotundone as one of the most potent known odorants (Siebert et al. 2008; Wood et al. 2008b). Interestingly, approximately 20% of the panelists were unable to detect rotundone at a concentration of 4000 ng/L (250 times the detection threshold) (Wood et al. 2008b). This phenomenon, where certain portions of the population are highly insensitive to a particular compound, is known as ‘specific anosmia;’ while not very common, this behavior has been observed for other wine flavor and aroma compounds including β-ionone (raspberry/violet) (Barter et al. 2015). Knowing this, it is
possible to speculate that two consumers drinking the same wine may have drastically different sensory experiences (Wood et al. 2008b).

Beyond this initial work, several studies have focused on understanding the consumer and expert perceptions and preferences of wines containing this ‘peppery’ aroma. Lattey et al. (2010) studied the sensory attributes that drive consumer and expert acceptance of Shiraz and Cabernet Sauvignon wines. The ‘pepper’ descriptor was found to be one of the most significant attributes negatively influencing consumer wine preferences. However, when the quality of the same wines was judged by expert winemakers, ‘pepper’ was considered a positive attribute as this descriptor was frequently noted in the richly flavored wines that were given higher quality scores (Lattey et al. 2010). Sensory trials were conducted to determine if and how the presence of rotundone (black pepper), guaiacol (woody), and eucalyptol (mint, camphor, eucalyptus) affected consumer acceptance and preferences of red wine (Herderich et al. 2012; Osidacz et al. 2010). Each aroma compound was individually spiked into a Merlot base wine at moderate and high concentrations. One hundred and four consumers were asked to rate the liking and purchase intent for the six spiked wines and the Merlot base wine. Looking at the overall liking scores, the wines with rotundone additions were liked moderately. When the data was split into three clusters based on liking patterns, it was determined that the addition of rotundone was positive for one-third of the consumers with the remaining two-thirds feeling relatively neutral towards its addition (Herderich et al. 2012; Osidacz et al. 2010). Geffroy et al. (2016) investigated the sensory and chemical characteristics of 21 Gamay wines from various locations within the Côtes d’Auvergne region (protected designation of origin, PDO) of France. Again, black pepper aroma intensity was positively related to rotundone concentration in the wine. Ethanol content was also positively correlated with rotundone, perhaps due to a greater extent of ripening and/or enhanced extraction via higher ethanol concentrations. A consumer ranking study of four of these wines showed that there was clear opposition between groups who preferred ‘peppery’ aromas versus
those who preferred ‘amylic’ aromas. The panelist demographics showed that many of the panelists who preferred the ‘peppery’ wines were managers and professionals who were willing to spend more for a bottle of wine (Geffroy et al. 2016).

Additionally, studies have been conducted in order to better understand the relationships between grape and wine compositional and sensory characteristics and black pepper aroma. Mayr et al. (2014) characterized the aroma profile of two distinctive types of high-priced Australian Shiraz wines, one from the cool climate Margaret River and one from the warmer Barossa Valley. The volatiles that were identified within the high-priced wines were spiked into model wine systems at the respective concentrations determined through instrumental analysis. A descriptive panel determined that the wine reconstitution samples, containing all key odorants, were in good agreement with the actual wine samples from the bottle. When rotundone was omitted from the reconstituted matrix, the dark fruit character was more pronounced, suggesting a black pepper masking effect. Additionally, when nonvolatile compounds (organic acids, sugars, minerals, and glycerol) were omitted from the reconstitution matrix, the pepper aroma intensity ratings were lower (Mayr et al. 2014). Mantilla et al. (2015) conducted a descriptive analysis study to identify relationships between sensory attributes and compositional measures of Shiraz grapes and wine. Firstly, the combination of four fruit attributes could predict wine savory spice flavor (cracked black pepper), with pulp prune and fresh fig flavors and seed bitterness being negative predictors for spice, while skin acidity was a positive predictor. The combination of berry pulp acidity, pulp detachment from skin, seed astringency, and seed bitterness could predict wine rim color, fresh dark berry flavor, and savory spice. Pulp acidity, seed astringency, and seed bitterness were found to be negative predictors while pulp detachment was a positive predictor for the described wine attributes. Furthermore, a negative relationship was observed between seed bitterness and wine savory spice, while pulp detachment from the skin was positively related to savory spice flavor (Mantilla et al. 2015).
1.7.8 Methodology for Rotundone Analysis

The first major challenge in establishing a method for rotundone analysis is that the (-)-rotundone and d<sub>7</sub>-rotundone standards are not commercially available, requiring synthesis by a skilled chemist (Cullere et al. 2016). Identification and quantification of sesquiterpenes can also be a daunting task due to similar elution times and mass spectrum profiles (Black et al. 2015). Much of the methodology currently available in the literature for rotundone analysis is very tedious as many sample preparation steps are necessary for isolation of the target compound (Cullere et al. 2016). Several chemical techniques have been employed in order to isolate, concentrate, detect, and quantify rotundone in grapes and wine. These techniques include stable isotope dilution analysis (SIDA), solid phase extraction (SPE), stir bar sorptive extraction (SBSE), solid phase microextraction (SPME), and various gas chromatography-mass spectrometry configurations (GC-MS, MDGC-MS, and GC-MS/MS).

Stable isotope dilution analysis is an accurate, precise, and robust method for the quantification of a target compound within a complex matrix such as wine. SIDA is a form of the internal standard method, and it allows for the analyte to be quantified based on the signal ratio between the internal standard and compound of interest. When selecting an internal standard for this analysis, it is important that it exhibits similar properties to the target compound without being inherently present in the sample matrix. The use of an isotopically labeled form of the analyte is ideal as it possesses almost identical properties to the target, and the two species can be effectively separated chromatographically (Hayasaka et al. 2005). The majority of the published literature for rotundone analysis has employed the use of a deuterated rotundone isotope as the internal standard. The most recent method developed for this analysis made use of β-damascone as it has a similar chemical structure to rotundone, is commercially available, and is not commonly found in wine (Cullere et al. 2016).
Following publication of the first protocol for rotundone analysis by Siebert et al. (2008) at the AWRI, many researchers have developed alternative methodology approaches in an effort to increase efficiency, sensitivity, and selectivity. The parameters for extraction of rotundone from homogenized grape samples have been modified by several researchers. In the Siebert et al. method, grape berry samples were homogenized, extracted with ethanol/water (2:3), and the extract was separated from the solids via sonication, centrifugation, and filtration. This filtrate was then spiked with the deuterated internal standard prior to SPE. Caputi et al. (2011) processed berries into a fine powder to increase surface area and reduce the length of extraction. Acetone, a more nonpolar solvent than model wine, was used as the extraction solvent to further increase efficiency. These method modifications allowed for a three-fold higher concentration of rotundone to be extracted and detected as compared to the method of Siebert and co-workers. Scarlet et al. (2014) and Zhang et al. (2015a) adapted Siebert’s protocol by separating the juice from the solids and performing a more rigorous extraction (50% v/v ethanol) of the berry skins, adding the juice back before performing SPE. Takase et al. (2015) extracted their berry samples with n-pentane/ethyl acetate (9:1) as this solvent system yielded the largest rotundone peak abundances of those trialed for this extraction. Quite possibly the most interesting approach for determination of rotundone concentrations in fruit was that employed by Geffroy et al. (2014; 2015b). Micro-fermentations were carried out, producing a small volume of wine from each fruit sample collected throughout the growing season; this methodology was implemented solely due to restrictions and logistical issues with shipping frozen grape samples from France to Australia for analysis.

For rotundone analysis in wine, most of the published literature used the protocol outlined in Siebert et al. (2008) in which 100 mL of wine is spiked with internal standard and used directly for SPE-SPME-GC-MS. Takase et al. (2015) reduced the amount of sample volume required for this analysis to 5 mL when using their SBSE method. More
recently, Cullere et al. (2016) developed a rapid determination method by SPE-GC-MS with liquid injection, which requires half the amount of wine as the Siebert method.

Solid phase extraction is a technique used to isolate a target compound from other components within a complex matrix and to concentrate the sample prior to instrumental analysis (Caputi et al. 2011). In this preparation method, efficient extraction and successful isolation of the compound of interest is highly dependent upon sorbent and solvent selection (Siebert et al. 2008). While many of the published methods successfully used SPE for sample preparation, Takase et al. (2015) used SBSE as an alternative to SPE in an effort to reduce the sample volume required for analysis and to avoid frustration over grape sample extracts causing clogged SPE tubes.

Solid phase microextraction (SPME) is a preparation technique that uses a sorptive fiber (absorptive and/or adsorptive) to sample, extract, and concentrate the analyte before instrumental analysis. While only a portion of the analyte is extracted by the fiber, the fraction sampled is proportional to the overall concentration of this compound within the sample (Petronilho et al. 2014). Seibert et al. (2008) investigated several SPME parameters in an effort to optimize the extraction of rotundone. Most notably, the use of direct immersion SPME yielded rotundone recoveries several orders of magnitude higher than headspace SPME most likely due to the low volatility of rotundone. Takase et al. (2015) preferred the use of SBSE over SPME as higher sensitivities could be achieved considering the larger amount of sorptive polydimethylsiloxane (PDMS) phase allows for more rotundone extraction than is possible with a SPME fiber.

For the detection and quantification of rotundone, Siebert et al. (2008) used GC-MS with selected ion monitoring (SIM). The method calibration curves for both grapes and wine showed excellent linearity with a quantitation limit of 0.5 ng/L; furthermore, repeatability experiments showed little variation as relative standard deviations (RSD) were less than 3%. Multidimensional gas chromatography-mass spectrometry (MDGC-MS)
methods were then developed for enhanced chromatographic separation to minimize the possibility of rotundone interfering with other components within the complex sample matrix. Validation of these methods showed good linearity across the concentration range, low limits of quantitation, and results were reproducible with little variation (Geffroy et al. 2014; Takase et al. 2015). Several groups also developed methods using gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) operated in multiple reaction monitoring mode (MRM) in order to enhance sensitivity and selectivity in the analysis of this trace compound. Once again, these methods showed an excellent calibration correlation with low limits of quantitation and high reproducibility (Caputi et al. 2011; Mattivi et al. 2011a).

1.7.9 In Oak Aged Spirits

A study conducted at the University of Illinois aimed to identify the compound responsible for the ‘woody/incense’ flavor in oak aged spirits (Genthner 2014). The target odorant was isolated from oak wood chips, and it was identified as rotundone by GC-MS-O. Following identification, rotundone concentrations in oak aged spirits were quantified and relationships between rotundone concentration, spirit type and aging time were explored. The oak aged spirits that were analyzed included seven bourbons, ranging from 4 to 12 years of aging, rye whiskey, Tennessee whiskey, scotch whiskey, aged rum, and añejo (aged) tequila. The lowest rotundone concentrations in these aged spirits were found in a scotch whiskey (0.150 µg/L) and aged rum (0.152 µg/L), with these low concentrations being attributed to the fact that these products were aged in previously used barrels. Legally, bourbon and rye whiskeys have aging requirements, which allowed for comparisons to be made based on the extent of aging and rotundone concentration. When several bourbons from the same manufacturer were analyzed, a positive relationship was observed between rotundone concentration and aging time. For instance, Jim Beam bourbons showed an increase from 0.342 µg/L (4 years) to 0.403 µg/L (8 years) to 0.453
μg/L (12 years). Bulleit whiskeys were found to have the highest rotundone concentrations of all of the spirits surveyed; rotundone concentrations in their bourbons were 0.694 μg/L (6 years) and 1.350 μg/L (10 years) and their rye whiskey (aged 4 years) had a concentration of 0.434 μg/L. This observation may indicate that manufacturing conditions, other than aging duration, may be important in the extraction of rotundone from the barrel, such factors may include species and origin of the barrel wood, temperature and humidity during aging, and barrel size. Through this work, rotundone was also identified in unaged silver tequila, suggesting that this compound may be naturally present in agave leaves. When the odor activity values of rotundone were compared to those of other volatiles present in bourbon, it was determined that rotundone does, in fact, have an impact on the overall flavor of bourbon whiskey (Genthner 2014).

1.8 Interspecific Hybrid Grape Varieties

Interspecific hybrid grape varieties are produced through the crossing of two or more Vitis species (Pedneault et al. 2013; Slegers et al. 2015). Many grape breeding programs across the world focus on hybrid cultivars in an effort to create new varieties with superior resistance to abiotic and biotic stress factors (Liu et al. 2016). For example, the development of cold-hardy hybrid varieties has made winemaking possible in cold climate regions such as the northern United States and Canada (Pedneault et al. 2013; Slegers et al. 2015). The genetic makeup of these interspecific vines allows for cold tolerance to extreme winter conditions, including temperatures as low as -30°C (Pedastsaar et al. 2014; Pedneault et al. 2013). In an effort to fulfill the demand for locally grown and sustainable wine production, grape breeding programs in these regions seek to create crosses that are not only cold-hardy but also are disease tolerant and have high enological potential (Slegers et al. 2015). By enhancing the tolerance for fungal diseases, nematodes, and phylloxera in these varieties, the need for chemical pest control treatments in the vineyard may be reduced (Pedastsaar et al. 2014).
The production of wine from hybrid varieties has proven to be successful in the Midwestern and Northeastern United States despite its relatively new emergence as it contributed over 400 million dollars to these economies in 2011 (Slegers et al. 2015). Still, this industry faces many challenges within the global market as these crossed varieties have a distinctly different fruit chemical composition from *Vitis vinifera* species. The higher anthocyanin content yields more intense colors with prominent blue tones. Tannins are typically low in concentration and degree of polymerization and exhibit poor extractability, presenting a challenge for winemakers. Titratable acidity levels are often high as is characteristic of grapes grown in cool climates. Wines produced from hybrid varieties possess a unique sensory profile, which may be associated with low quality, making them susceptible to criticism by members of the wine community. This undesirable ‘hybrid’ aromatic character is thought to be linked to ‘grapey’ and ‘foxy’ compounds, including methyl anthranilate, furaneol, and o-aminoacetophenone, which are native to *V. labrusca* varieties. The effects of viticultural and winemaking practices on the chemical and aromatic profiles of these hybrid varieties produced in northern climates are still poorly understood. Further research in these areas may allow for a better understanding of the impact of such cultural practices on hybrid flavor and aroma, allowing for optimization of grape and wine quality (Slegers et al. 2015).

### 1.9 The Noiret Variety

The Noiret (pronounced *nwahr-ay*) variety was released by Cornell University in 2006. This complex interspecific red hybrid of *Vitis* species is a cross of NY65.0467.08 (NY33277 x ‘Chancellor’) and ‘Steuben’ (Reisch et al. 2006; Robinson et al. 2012). The first vines of Noiret were planted in 1975, and researchers began testing its suitability for winemaking in 1980.

Noiret has large, loose clusters composed of large berries (Reisch et al. 2006; Robinson et al. 2012). Average yields for Noiret (5.6 kg/vine) tend to be lower than
Concord (7.6 kg/vine) (Vanden Heuvel et al. 2013). This variety produces large vines and exhibits significantly higher pruning weights than other hybrid varieties. Furthermore, Noiret vines yield low crop load ratios (fruit mass/pruning weight) as they are often overly vegetative and therefore produce low crop yields (Vanden Heuvel et al. 2013). Own-rooted Noiret vines are capable of surviving in phylloxera infested soils (Reisch et al. 2006). This red hybrid is moderately cold hardy and somewhat susceptible to downy mildew and black rot, while Botrytis incidence is uncommon. Noiret is late bud bursting and exhibits mid to late ripening with veraison generally taking place in early September and harvest between late September and early October under the climatic conditions of Upstate New York (Reisch et al. 2006; Robinson et al. 2012).

Noiret juice has a deep, red color. Acid adjustments are not typically required prior to fermentation as high acidity and pH are not a concern; in fact, the wine acidity tends to be well-balanced following MLF. Sugar additions to the must may be necessary to achieve a TSS content of 20 to 22 °Brix prior to initiation of primary fermentation. Noiret wines exhibit a fine tannin structure and mouth-filling texture with consistency and richness from the front to the back of the palate (Reisch et al. 2006). The variety’s aroma profile contains raspberry and blackberry fruit, mint, and notes of green and black pepper, and it lacks the ‘hybrid’ aromas that are often associated with other hybrid varieties (Reisch et al. 2006; Robinson et al. 2012).

1.10 Purpose and Significance

In 2014, 24 billion liters of wine were consumed across the world; the United States alone accounted for 3.1 billion liters of this total, making the United States the top wine consuming country (Wine 2015). The United States ranks second in grape production, contributing 21% of the total world grape production with 55% of those grapes being used for wine. Additionally, the U.S. is the fourth largest wine producing country, providing 8% of the global market (Wine 2015). Within the U.S., the grape and wine industries have an
estimated economic impact of $162 billion on the U.S. economy (LLC 2007). Within Pennsylvania, these industries have a combined economic value of approximately $1.9 billion with the wine industry alone contributing roughly $980 million (LLP 2013). It is generally recognized that the continued growth of the wine industry is dependent upon the ability to consistently produce high-quality wine grapes (Owens 2008; Zoecklein et al. 1999).

Premium wine production is associated with the optimum balance of flavors and aromas. Improving flavor and aroma potential and balance in wine grapes is a priority for many growers across the world. The knowledge garnered through recent research in the fields of enology and viticulture has allowed for increased understanding of specific flavor and aroma compounds which contribute to, or detract from perceived wine quality (Allen and Lacey 1998). Currently, however, there is a poor understanding of how viticultural practices impact the development of specific flavor and aroma compounds that contribute to wine quality. Consistently, these vineyard management practices are being evaluated based on their impact on TSS, pH, and TA, without consideration of aroma potential due to the fact that these parameters are often used as the main indicators of ripeness and quality in the field.

Much of the research surrounding the newly discovered aroma impact compound rotundone has focused on analytical methods for isolation and detection in grapes and wine. After rotundone was identified as a grape-derived aroma compound, many researchers began studying the inherent environmental and viticultural factors impacting rotundone accumulation in the fruit in an effort to better understand its mechanism of formation. Beyond the impacts of environmental and cultural factors on variations in the black pepper character in wine grapes, many have suggested that vineyard management practices can be used to alter rotundone concentrations to the desired level in the vineyard. Previous work on the impact of light availability to the fruit on rotundone development found inconsistent results. Most of these studies did not evaluate whether leaf
pulling resulted in canopy density and light availability differences as compared to undefoliated vines; therefore, it is uncertain whether these treatments were effective enough to elicit differences in fruiting microclimate. Furthermore, previous work has not explored the impacts of the duration or the timing of fruit sunlight exposure on rotundone formation.

Production of quality wines from hybrid wine grape varieties is often difficult due to the so-called 'hybrid aroma' that is commonly associated with low-quality wine. Noiret is a cold-climate interspecific red hybrid variety which is grown in the northeastern United States. This variety lacks typical hybrid character and therefore has the potential to produce quality varietal wines. Winemakers and experienced tasters have anecdotally noted that the Noiret variety possesses the spicy, black pepper aroma and flavor that is characteristic to rotundone, yet the presence of this compound has not yet been confirmed via analytical chemistry methods. Thus far, research investigating the development of rotundone in wine grapes has predominantly been conducted in Australia, New Zealand, Italy, and France. Therefore, this compound has not been identified in many wine grape varieties grown in the United States, and the dynamics of its development have not been studied within the scope of the northeastern United States climate.

Sensory analysis of Noiret wines vinified from the different vineyard treatments may also provide insight on the impact of viticultural practices which affect fruit zone microclimate on black pepper aroma intensities in the wines. From an economic perspective, understanding if and how cluster sunlight exposure and the timing of basal leaf removal impact rotundone accumulation in grapes and its concentration in wine may allow for considerable savings in labor costs by reducing unnecessary, or even counterproductive efforts, to manipulate this compound in the vineyard.
1.11 Hypotheses and Objectives

I hypothesize that rotundone is responsible for the spicy, black pepper aroma characteristic to the Noiret wine grape variety. I also hypothesize that rotundone concentrations in Noiret grapes and wine will be reduced when viticultural practices allow for increased sunlight exposure to reach the fruiting zone during the ripening stage of development. Finally, I hypothesize that black pepper aroma intensity ratings will positively correlate with rotundone concentrations in the wines vinified from the viticultural treatments.

To test these hypotheses, the following objectives were established:

1. Identify the presence of rotundone in Noiret grapes and wine
2. Determine if and how the timing and duration of cluster sunlight exposure affect rotundone development and concentration in the fruit and the perceived black pepper aroma intensities in the resulting wines
3. Evaluate the correlation of quantitative measures of cluster sunlight exposure, fruit yield, vine vigor, and fruit composition to the concentration of rotundone in Noiret grapes
4. Determine the relationship between perceived black pepper aroma intensities and rotundone concentrations in Noiret wine
Chapter 2

Materials and Methods

2.1 Vineyard Site and Experimental Design

The experiment was conducted from 2014 to 2015 at the Cornell University New York State Agricultural Experiment Station (NYSAES) in Geneva, NY, U.S. (42°N, 77°W). The vineyard soil is deep, well-drained Honeoye fine silt loam. The vines, interspecific red hybrid Noiret (NY65.0467.08 x Steuben), were grown on a high-wire cordon trellis system with vine spacing of 3.6 m between vines, 2.7 m between rows, and oriented north-south. The experiment was designed using a randomized complete block design with five replications per treatment. Each experimental unit was composed of a single panel containing two vines.

Two treatments were implemented on own-rooted Noiret vines to assess the effect of the intensity and duration of sunlight exposure on rotundone development: 1) control (CON) (no leaf removal, highly shaded clusters); 2) “maintained” 100% cluster sunlight exposure (MSE) (leaves continuously removed from the fruiting zone beginning at berry pea-sized stage to maintain sun exposure throughout the season). Two additional treatments were employed using Noiret vines grafted on Millardet et de Grasset 101-14 (101-14 Mgt) rootstock to assess the impact of the timing of basal leaf removal on rotundone development: 3) pre-veraison leaf removal (LR) (basal leaves removed at berry
pea-sized stage); 4) post-veraison leaf removal (PVLR) (basal leaves removed one week after 50% veraison). Basal leaf removal for treatments 3 and 4 was only carried out at the single specified time point; further leaf removal to maintain sun exposure in the fruiting zone was not implemented. Additionally, treatments 1 and 2 could not be compared to 3 and 4 due to potential variation that may arise from the two different rootstock types.

2.2 Canopy Characterization

To assess the impact of the treatments on canopy density and microclimate in the fruiting zone, point quadrat analysis (PQA) and light availability in the fruiting zone were measured three times during the growing season. For point quadrat measurements, a tape measure was first extended across each treatment panel parallel to the fruiting zone. A thin metal rod was inserted into the canopy at 20 cm intervals (36 insertions per panel), and the order in which the clusters and leaves were contacted was recorded (Meyers and Vanden Heuvel 2008). Photosynthetically active radiation (PAR) measurements were taken in the fruiting zone using a ceptometer (Decagon Devices, model AccuPAR LP-80, Pullman, WA).

For the 2014 season, these measurements were acquired: 21 days after the first leaf removal event was imposed on MSE and LR vines (6 Aug), eight days after late leaf removal was implemented (12 Sep), and 21 days before harvest (7 Oct). During the 2015 season, these measurements were taken: 14 days after the first leaf removal event was imposed on MSE and LR vines (16 Jul), 15 days after late leaf removal was implemented (11 Sep), and 13 days before harvest (8 Oct).

PQA has consistently been used in viticulture to evaluate canopy microclimate. More recently, an enhanced form of this analysis was developed to incorporate percent photon flux (PPF) measurements for determination of light exposure to the clusters and leaves. The PQA and PAR data were analyzed using Enhanced Point Quadrat Analysis (EPQA) and Calibrated Exposure Mapping (CEM) Tools (version 1.6.2) (Meyers and
Vanden Heuvel 2008). Through these tools, several quantitative parameters relating to canopy density and light availability to the fruit were calculated. As leaves are pulled from the vines, one would expect to observe an increase in percent gaps (PG) as well as cluster and leaf exposure flux availability (CEFA, LEFA). Pulling foliage from the vines would result in more recorded gaps with less overall cluster/leaf contacts and light (flux) would be more available to the clusters and remaining leaves. Additionally, this management practice would be expected to decrease leaf layer number (LLN), percent interior clusters and leaves (PIC, PIL), occlusion layer number (OLN), and cluster and leaf exposure layer (CEL, LEL) as these parameters measure canopy density and positioning within the interior of the canopy as opposed to the outer, exposed portion of the canopy.

2.3 Berry Sampling and Yield Components

Two hundred and fifty gram berry samples were collected from each experimental unit at four time points throughout the growing season: before veraison, bunch closure phenological stage (22 Jul 2014 and 29 Jul 2015); at 100% veraison (1 Sep 2014 and 27 Aug 2015); midway between veraison and harvest (26 Sep 2014 and 25 Sep 2015); and at harvest (28 Oct 2014 and 21 Oct 2015). The berry samples were placed in plastic storage bags, kept on ice during transport, and stored at -20°C until analysis.

Grapes were hand-harvested on 28 Oct 2014 and 21 Oct 2015. At this time, clusters were counted for each experimental unit, and crop yield was assessed using a hanging scale with 0.1 kg accuracy (Rubbermaid, Inc., Pelouze 7710, Huntersville, NC). These measurements were used to determine average cluster weight, total crop weight, and number of clusters per vine. An additional 100 count berry sample was collected per experimental unit for determination of berry weight, pH, TA, and TSS.

The vines were pruned during the dormant season to determine vine vegetative growth from the previous year (3 Apr 2015 and 4 March 2016). The mass of the wood removed from each experimental unit was measured using a hanging scale with 0.1 kg
accuracy (Salter Brecknell, SA3N340, Fairmont, MN); these data were used to calculate the vine crop load, expressed as Ravaz index (yield/pruning weight).

Seasonal weather data (daily average, maximum, and minimum air temperature, leaf wetness hours, total rain, relative humidity, average wind speed, and solar radiation) and hourly temperature from veraison to harvest were obtained from the Cornell University Network for Environment and Weather Applications (NEWA) Geneva station (newa.cornell.edu). Growing degree days (GDDs) were determined from 1 May to harvest using GDDs = [(maximum daily temperature + minimum daily temperature) / 2] – 10.

2.4 Vinification

The harvested fruit was transported to the Department of Food Science at the Pennsylvania State University where it was stored less than 48 hours at 3°C until processing (30 Oct 2014 and 22 Oct 2015). Fruit weights were recorded for each treatment to estimate grape yields. The fruit was crushed and destemmed by treatment and was evenly distributed between two 40 L (2014) or 60 L (2015) open-top, low-density polyethylene fermentation bins (Nalgene Nunc International, Waltham, MA), yielding ~30 to 40 L of must per bin. Fifty milliliter samples of juice were taken from each treatment for TSS (°Brix) and yeast assimilable nitrogen (YAN) measurements. Both years, the must of each fermentation replicate was chaptalized with sucrose to achieve a TSS value of 21 °Brix based on the juice chemical analyses; however, acid adjustments were not carried out.

Each of the treatment bins was inoculated with a commercial strain of Saccharomyces cerevisiae ICV-GRE yeast (Lallemand, Petaluma, CA) at an inoculation rate of 0.25 g/L with additions of 0.30 g/L of Go-Ferm nutrient (Lallemand, Petaluma, CA). Primary fermentation was carried out without the use of external temperature control. Pomace caps were punched down three times per day, and the progression of alcoholic fermentation was monitored daily by temperature and TSS readings using an
electronic thermocouple (Fluke Corporation, Everett, WA) and hydrometer, respectively. YAN adjustments were performed at one-third sugar depletion with the addition of Fermaid K nutrient (Lallemand, Petaluma, CA) to achieve a final concentration of 0.25 g/L.

When the Brix measurements dropped below zero by hydrometry, Clinitest (Bayer AG, Leverkusen, Germany), was used to determine dryness, which was defined as < 1% residual sugar. At dryness, each wine treatment replicate was pressed and transferred into an 18.93 L glass carboy using a hydraulic, stainless-steel basket press. The press was sanitized between replicates of the same treatment and was thoroughly washed and sanitized between treatments. While pressing, 500 mL samples were taken from each replicate for pH, TA, alcohol, and volatile acidity (2015) measurements. The pressed wines were inoculated with a commercial strain of *Oenococcus oeni* Alpha MBR malolactic bacteria (Lallemand, Petaluma, CA) at a concentration of 10⁵ CFU/mL. Malolactic fermentation (MLF) was monitored weekly by paper chromatography.

At the completion of MLF, wine samples (200 mL) were taken from each fermentation replicate and were snap frozen and stored at -80°C until rotundone analysis could be performed. An additional sample aliquot (100 mL) was taken for pH, titratable acidity, alcohol, volatile acidity, free SO₂, total SO₂, and color density (2015) measurements. The wines were placed in cold storage (3°C) for two weeks to begin tartrate stabilization, after which point, the wines were racked off the lees. Treatment replicates were combined into a single 18.93 L glass carboy per treatment. At this time, sulfur dioxide was added to achieve a final concentration of 0.90 mg/L molecular free SO₂, adjusted according to the average pH of the combined treatment replicates. The wines were placed back into cold storage for continued stabilization. A second racking was performed at least one week prior to bottling and additional sulfur dioxide was added to achieve the desired molecular free SO₂ concentration of 0.90 mg/L. Existing free sulfur dioxide concentrations were determined one day before racking by the aeration oxidation
method. Wine treatments were manually bottled in sanitized 750 mL-capacity clear glass bottles and sealed with a screw cap. Bottled wines were maintained under cold storage until sensory evaluation and GC-MS analysis.

2.5 Berry and Wine Chemical Analysis

2.5.1 Berry Sample Chemical Analyses

Frozen berry samples were used to analyze mean berry weight, TSS, pH, and TA at the time of sampling. A 100 berry sub-sample from each sample bag was transferred to a beaker and the weight was recorded for berry weight determination. This subsample was placed back in the respective plastic sample bag and sealed. Each bag was placed into a water bath at 60°C in order to thaw and dissolve tartrates. The samples were then pressed in the bags to expel juice, strained through several layers of cheesecloth to remove solids, and collected in a beaker. Total soluble solids were measured using a handheld refractometer (Master, Atago, Nelleve, WA). pH was measured using an Orion Star A111 pH meter (Thermo Fisher Scientific, Waltham, MA). Titratable acidity was measured using an autotitrator (G20, Mettler Toledo, Columbus, OH). A 10 mL sample of juice was made up to 40 mL with deionized water, titrated to a pH of 8.2 using 0.10 N sodium hydroxide, and the results were recorded using tartaric acid equivalents.

2.5.2 Wine Chemical Analyses

Wine pH was measured as described above (Section 2.5.1). Prior to TA measurements, a wine sample aliquot was transferred to a labeled tube and heated in warm water (ca. 60°C) for 10 minutes. The samples were then purged with gaseous argon for 30 seconds in order to remove carbon dioxide. After purging, TA was measured using an autotitrator as described previously (Section 2.5.1). Alcohol content, volatile acidity, total and free SO₂ and color density were analyzed using standard wine analysis methods (Zoecklein et al. 1999). Alcohol content in the wines was determined using an ebulliometer.
Volatile acidity was measured using Cash still distillation and results were recorded as acetic acid equivalents. Total SO₂ measurements were performed using the ripper method. Samples were diluted 1:1 prior to analysis as the intense color did not allow for a noticeable color difference; the concentrations that were determined using the diluted sample were then multiplied by the respective dilution factor. Free SO₂ measurements were acquired using the aeration oxidation method. For color density measurements, the wines were diluted one in ten with ultrapure water adjusted to the pH of the wine sample. Absorbance readings were acquired using a UV-Vis spectrophotometer (Genesys 10S, Thermo Fisher Scientific, Waltham, MA) at wavelengths 420, 520, and 620 nm.

2.6 Chemicals

Rotundone and d₅-rotundone standards were generously donated by researchers at the Australian Wine Research Institute. Working solutions of these standards were prepared volumetrically in high purity ethanol and were stored at -20°C until use. High-performance liquid chromatography (HPLC) grade pentane was purchased from Fisher Scientific (Waltham, MA). HPLC-grade methanol and ethyl acetate, 200 proof ethanol, and reagent grade acetone were all obtained from VWR International (Radnor, PA) L-Tartaric acid was purchased from Sigma-Aldrich (St. Louis, MO). Water was purified using a Millipore Q-Plus system (Millipore Corp., Bedford, MA).

2.7 Sample Preparation for Rotundone Analysis

2.7.1 Grapes

One hundred and twenty-five grams of berries from each frozen grape sample (-20°C) were deseeded and the skins and pulp were carefully transferred to a dewar of liquid nitrogen. The liquid nitrogen and berries were stirred with a spatula. Any remaining liquid
nitrogen was subsequently discarded. The frozen berries were transferred to a 14-speed kitchen blender (Sunbeam Products, Inc., Boca Raton, FL) and were homogenized on the high chop setting until a fine powder was achieved. The frozen berry powders were transferred to labeled glass sample jars. The headspace above the sample was purged with argon for 30 seconds prior to sealing with the cap. The sample jars were placed in frozen storage (-80°C) until analysis.

Twenty-five grams of frozen grape powder was spiked with 100 µL of the d₅-rotundone (516 µg/L in ethanol) internal standard. Fifty milliliters of acetone was added to the spiked berry powder and the mixture was shaken orbitally at 225 rpm for 1 hr. The extracts were vacuum filtered using 0.10 µm glass fiber filter paper (Pall Corporation, Port Washington, NY). The solvent was carefully evaporated at 40°C using a nitrogen blow down RapidVap Vertex+ Dry Evaporator (Labconco Corp., Kansas City, MO). The remaining aqueous residues (ca. 20 mL) were diluted to 85 mL with model wine (12% ethanol, 5 g/L tartaric acid, pH 3.2). The diluted samples were distributed across two Teflon fluorinated ethylene propylene (FEP) centrifuge tubes (Nalgene Nunc International Corp., Rochester, NY) and were centrifuged at 4000 rpm for 12 minutes. The centrifuged extract was used for SPE analysis.

SPE cartridges (Phenomenex Strata styrene-divinylbenzene (SDB-L) 500 mg/6 mL) were conditioned with one cartridge volume of n-pentane/ethyl acetate (4:1), followed by methanol, and finally, model wine. The berry extract/wine sample was then loaded onto the SPE cartridge, which was subsequently washed with a cartridge volume of ultrapure water followed by n-pentane (2 mL, discarded). Elution was carried out using two 5 mL aliquots of n-pentane/ethyl acetate (9:1). The two sample fractions were evaporated to dryness using a nitrogen blow down evaporator at 40°C. The remaining dried residues were reconstituted in ethanol (0.5 mL) and ultrapure water (6.5 mL) was added. The reconstituted liquid was transferred to a 10 mL GC vial with magnetic screw cap for analysis by SPME-GC-MS.
2.7.2 Wines

For wine samples, 100 mL of the wine was directly spiked with 100 μL of d₆-rotundone (516 μg/L in ethanol) prior to SPE which was carried out as described previously (Section 2.7.1) with minor modifications. The dried eluate was reconstituted in ethanol (0.5 mL) and ultrapure water (4.5 mL) was added. The sample was then poured into a carefully labeled 10 mL GC vial with a magnetic screw cap. Sample vials were placed in frozen storage (−80°C) until analysis. Once prepared, the wine extracts were packaged with Techni Ice packs (frozen at -80°C) and sent to the Australian Research Wine Institute (Urrbrae, South Australia) for analysis.

2.8 Rotundone Quantitation in Whole Berry Extracts by SPME-GC-MS

2.8.1 Instrument Parameters

An Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA) equipped with a Gerstel MPS2 multipurpose autosampler (Gerstel GmbH &Co. KG, Mülheim an der Ruhr, Germany) was coupled to an Agilent 5975C mass selective detector. Agilent MassHunter GC/MS Acquisition software (Version B.07.00 SP2.1654) and Gerstel Maestro 1 software (Version 1.3.20.41) was used to control the instrument and autosampler, respectively. The GC was fitted with a J&W VF-35ms capillary column (60 m x 0.25 mm x 0.25 μm). The GC inlet was fitted with a silanized glass straight design inlet liner (78.5 mm long x 6.5 mm o.d. x 0.75 mm i.d.) (Supelco, Bellefonte, PA). Helium (ultra high purity) was used as the carrier gas.

A Supelco “pink” StableFlex polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber was directly immersed in the sample for 60 min at 35°C with agitation (250 rpm). The SPME fiber was desorbed in the inlet of the gas chromatograph at 240 °C for 5 minutes. Pulsed pressure mode was used during injection with a pulsed pressure of 18.362
psi until 36 s. The split valve ratio was set to 30:1 after 33 s. The helium carrier gas was run using constant flow mode at a rate of 1 mL/min. The oven temperature was first held at 80°C for 1 minute, increased to 220°C at 3°C/min, followed by an increase to 240°C at 40°C/min, and finally was maintained at 240°C for 20 minutes. The MS transfer line was held at 240°C. The MS source and quad were operated at 230°C and 150°C, respectively. Positive ion electron impact selected ion monitoring (SIM) at 70 eV was used for the determination of rotundone and d₅-rotundone. The monitored ions were m/z 147.1, 161.0, 203.1, 208.1, 218.1, and 223.2 with a dwell time of 30 ms for each. Rotundone was quantified using m/z 218.1 as the target ion and m/z 203.1 as the qualifier, and d₅-rotundone was quantified using m/z 223.2 as the target ion and m/z 208.1 as the qualifier. Data analysis was performed using the Agilent MDS Enhanced ChemStation software package (Version F.01.00.1903).

2.8.2 Method Calibration and Repeatability

Approximately two kilograms of black seedless table grapes (STEVCO, Beverly Hills, CA) were crushed in large plastic bags to expel their juice, the juice was separated from the skins, and samples were taken for Brix, pH, and TA measurements. The juice chemistries were then adjusted to reflect the average harvest chemistries for Noiret grapes grown in the Finger Lakes AVA (NY) between 2010 and 2014 (Martinson 2014). The TSS were adjusted first from the initial value of 19.2 °Brix to 18.2 °Brix via dilution with deionized water. The pH of this diluted juice was measured and then adjusted from 3.25 to 3.35 using a 10 N sodium hydroxide solution. No adjustments were made to TA (7.55 g/L tartaric acid) following the Brix and pH adjustments. The modified juice was recombined with the grape skins prior to freezing in liquid nitrogen and grinding into a powder as described previously. Subsamples of this grape powder were spiked in duplicate with rotundone at concentrations: 0, 0.20, 0.80, 1.37, 1.94, 2.75, 3.20, 3.66, and 4.23 μg/kg (n = 9 x 2). The calibration curve exhibited good linearity (r² = 0.992) across the concentration
range. The reproducibility of this method was investigated using several replicate additions of rotundone at a low (0.20 µg/kg) and a high (1.94 µg/kg) concentration within the calibration range. Both concentrations showed RSD values of < 3%. Several replicate additions of 0 and 0.057 µg/kg of rotundone were analyzed for the determination of limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ for the method were calculated to be 0.049 and 0.163 µg/kg, respectively.

2.9 Rotundone Quantitation in Wines by SPME-MDGC-MS

2.9.1 Instrument Parameters

The established GC-MS method described in Section 2.8 did not allow for adequate separation of rotundone and the deuterated internal standard in wine extracts. This was most likely due to the evolution of additional volatile components during fermentation which resulted in peak interferences; thus, multidimensional gas chromatography was necessary to achieve separation of these components for quantification. Therefore, wine sample extracts were analyzed at the Australian Wine Research Institute using a previously established multidimensional gas chromatography-mass spectrometry (MDGC-MS) method.

The MDGC-MS apparatus was set up as described previously (Geffroy 2014). Cryotrap cooling was not employed for this analysis. Samples were incubated at 35°C for 0.50 min prior to extraction with a Supelco “blue” PDMS/DVB SPME fiber for 60 min at 35°C with agitation (250 rpm). The SPME fiber was desorbed in the GC inlet at 250°C for 5 min. Splitless mode was used, opening the splitter at 23:1 after 60 s. The flow of carrier gas through the D¹ column was held at a pressure of 46.5 psi for 50 min and then increased at a rate of 6 psi/min to a pressure of 77.5 psi, which was held for 0 min. The carrier gas flow through the D² column was held at a pressure of 34.95 psi for 50 min and then increased at a rate of 5 psi/min to 60 psi, which was held for 0 min. The oven temperature
was first held at 80°C for 1 min, increased to 215°C at 5°C/min, cooled to 130°C at
15°C/min and held for 2 min, then increased to 195°C at 5°C/min, and finally increased to
280°C at 40°C/min and held for 10 min. The MS transfer line was held at 250°C. The MS
source and quad were operated at 230°C and 150°C, respectively. The MS was operated in
positive EI mode at 70 eV using simultaneous scan/SIM mode. The scan used a mass
acquisition range of m/z 35 to 280. The ions selected for SIM determination of rotundone
and the d₇-rotundone internal standard were m/z 147.0, 161.0, 163.0, 203.0, 208.0, 211.0,
218.0, and 223.0, and 226.0 with a dwell time of 25 ms for each. Rotundone was quantified
using m/z 218.1 as the target ion and m/z 203.1 as the qualifier, and d₇-rotundone was
quantified using m/z 223.2 as the target ion and m/z 208.1 as the qualifier. Data analysis
was performed using the Agilent MDS Enhanced ChemStation software package (Version
F.01.00.1903).

2.9.2 Method Calibration

A calibration curve for this method was constructed by spiking a Cabernet
Sauvignon wine with rotundone in duplicate at concentrations of 0, 200, 400, and 629 ng/L
(n = 4 x 2). These samples yielded a linear relationship across the calibration range (r² =
0.999).

2.10 Sensory Analysis

2.10.1 Panelist Training and Discrimination Test

Sensory testing was conducted at the Cornell University Sensory Evaluation Center
(Ithaca, NY). Sixty-five panelists, consisting of university faculty, students, and staff
between the ages 18 and 64, were recruited for the training and discrimination task which
was held on 10, 11, and 14 Mar 2016. Panelists were seated in individual booths without
the use of red light. First, panelists were asked to smell and describe the aroma of a
ground black pepper standard (ca. 2.0 g, presented in a capped clear plastic container).
Following description, panelists were told that this descriptor corresponded to black pepper aroma. Next, panelists were presented with a triangle difference test using the MSE and LR wines from the 2014 vintage. These samples were chosen as the sensory program manager determined that the wines had noticeably different intensities of black pepper aroma. Thirty milliliters of each wine was poured into standard ISO glasses, covered, and labeled with four-digit codes. The samples were presented at a temperature of 20°C ± 2°C. Each panelist was given one test, consisting of three wines, and was asked to smell each wine and identify which sample was different by olfaction analysis. With 65 panelists completing the test, 29 correct judgments were required to establish significance ($\alpha = 0.05, \beta = 0.05, P_{max} = 40\%$).

2.10.2 Black Pepper Intensity Descriptive Analysis

A descriptive analysis test of the 2014 and 2015 Noiret wines was conducted on 15 Mar 2016 at the Cornell University Sensory Evaluation Center (Ithaca, NY). Panelists were chosen based on participation in the training and discrimination test as well as whether a correct description was given for the ground black pepper standard. This panel was comprised of 61 individuals (41 females and 20 males, ages 21 to 64) who reportedly drink wine at least a couple times a year. Due to the strong black pepper aroma present in the wines, which may cause an adaptation effect, different dilution ratios were tested, and it was determined that black pepper intensity differences between treatments were more evident when the wines were diluted. Therefore, the bottled Noiret wines were diluted, mixing two parts wine with one part deionized water (2:1 v/v). Panelists completed the evaluation in individual booths without the use of red light. Thirty milliliters of dilute wine were dispensed into standard ISO glasses, capped, and labeled with four-digit codes. The samples were presented at a temperature of 20°C ± 2°C. Panelists were asked to assess 16 wines (four treatments x two vintages x two replicates) during one testing session with each wine presented individually. The presentation order was randomized, with one
replicate set (four treatments x two vintages) being presented in random order first immediately followed by the second replicate set in random order. Panelists were asked to smell each wine and rate the black pepper aroma intensity on an unstructured line scale, with anchor points of “not at all” and “extremely strong” placed at 0 and 100%, respectively. RedJade sensory analytics software (Tragon Corporation, Redwood Shores, CA) was used for data collection.

2.11 Statistical Analysis

Statistical analyses were performed using SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC). Statistical comparisons between the respective sun exposure and leaf removal treatments were performed using Proc Mixed (α = 0.05, 0.10) procedure with replicate coded as a random effect. For correlation determinations, Proc Reg (α = 0.05) was used to perform linear regression analysis. Data from the 2014 and 2015 seasons were not combined over years due to significant treatment by year interactions for the rotundone data.
Chapter 3

Results and Discussion

3.1 Fruit Zone Canopy Density and Light Availability

Enhanced point quadrat analysis was used to assess the effects of the leaf removal treatments on canopy density and cluster light availability to the fruit throughout the growing season. In 2014, no differences in EQPA parameters were observed between either treatment comparison on the first measurement date (6 Aug) (Table 3.1). This was unexpected as leaf removal was previously implemented on the LR and MSE vines. It is possible that the lack of differences observed between treatments was a result of not enough leaves being pulled around the fruit of the LR and MSE vines. In this pre-veraison stage, the vines are also in their highly vegetative growth stage; it may be possible that significant vegetative growth occurred during the 21 days between leaf pulling and EPQA data collection, resulting in not significant differences between treatments. Additionally, it should be noted that a hail storm hit the vineyard on 31 Jul 2014. This weather event resulted in severe damage to exposed fruit and leaves, which also may have contributed to the lack of differences in canopy characteristics on the first EPQA data collection date. The second EPQA measurement was 11 days after veraison and eight days after the PVLR treatment was employed. At this time, comparison of the canopy characteristics showed the expected trends as the MSE vines had less dense canopies (PG, LLN, OLN,
CEL, LEL), less interior clusters and leaves (PIC, PIL), and more light available to clusters and leaves (CEFA, LEFA) than the CON, with each parameter exhibiting significant differences (Table 3.1). The timing of leaf removal treatments (LR vs. PVLR), however, did not significantly impact any EPQA parameters at this time. This was unexpected as PVLR was carried out shortly (eight days) before these measurements were conducted. The lack of differences may be attributed to the absence of lateral shoot growth in the fruiting zone in the LR treatment, possibly due to damage to the lateral buds during the hail storm. The final EPQA measurements were taken 21 days prior to harvest. Significant differences were observed between the sun exposure treatments (CON vs. MSE) for every parameter, except for PG, and the relative trends for each parameter aligned with those of the previous data collection. Again, the timing of leaf removal (LR vs. PVLR) did not significantly impact EPQA parameters; this would be expected as further leaf removal was not carried out in either treatment following the second EPQA data collection.

During the 2015 season, differences in EPQA parameters between the CON and MSE treatments were maintained and similar to those reported for the 2014 season. Comparisons of these two treatments showed significant differences at all three data collection points for all of the EPQA parameters, except for PG on the first and second dates (Table 3.2). Differences in canopy density and light availability parameters between the timing of leaf removal treatments (LR vs. PVLR) changed over the season. In 2015, the first EPQA sampling date was carried out 14 days after leaf removal was applied on the LR vines. As expected, the LR treatment decreased the layers of leaves (LLN) and degree of shading (LLN, CEL, LEL) as well as increasing the amount of light reaching the clusters and leaves (CEFA, LEFA) as compared to the PVLR vines. The second set of EPQA measurements were conducted 15 days after PVLR was implemented. EPQA metrics were significantly different between LR and PVLR except for LEL and LEFA; PVLR vines had less dense canopies (PG, LLN, OLN, CEL), less interior clusters (PIC)
and leaves (PIL), and more light intercepted by clusters (CEFA) than the LR vines. The changes of each EPQA parameter from time point one to two indicates an increase in canopy density and decrease in light availability for the LR vines. This suggests that vegetative growth of lateral shoots occurred in the fruiting zone. The final EPQA measurements for this season were taken 13 days prior to harvest, and LR vines had significantly denser canopies (LLN, CEL), more interior clusters (PIC), and less light available to the fruit (CEFA) and leaves (LEFA) than the PVLR vines. Looking at the changes in these parameters from time point two to three for the LR vines, most of the canopy density metrics (LLN, OLN, CEL, LEL) decreased and light availability (CEFA, LEFA) increased. Additionally, the PVLR treatment vines generally showed a decrease in PG, similar or slight increases for canopy density metrics, and increases in light availability from time point two to three. These changes may be due to foliage losses through natural leaf abscission.
Table 3.1: Effect of basal leaf removal treatments on Noiret vine canopy density and light availability in the fruiting zone during 2014.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PG</th>
<th>LLN</th>
<th>PIC</th>
<th>PIL</th>
<th>OLN</th>
<th>CEL</th>
<th>LEL</th>
<th>CEFA</th>
<th>LEFA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>August 6, 2014</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>2.22</td>
<td>3.66</td>
<td>79.3</td>
<td>49.1</td>
<td>4.20</td>
<td>1.38</td>
<td>0.71</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MSE</td>
<td>3.90</td>
<td>2.99</td>
<td>62.9</td>
<td>43.7</td>
<td>3.58</td>
<td>0.93</td>
<td>0.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.302</td>
<td>0.233</td>
<td>0.113</td>
<td>0.468</td>
<td>0.306</td>
<td>0.112</td>
<td>0.371</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LR</td>
<td>2.25</td>
<td>3.19</td>
<td>70.6</td>
<td>45.4</td>
<td>3.85</td>
<td>1.07</td>
<td>0.60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PVLRL</td>
<td>0.00</td>
<td>3.93</td>
<td>79.7</td>
<td>52.4</td>
<td>4.55</td>
<td>1.27</td>
<td>0.77</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.097</td>
<td>0.089</td>
<td>0.204</td>
<td>0.144</td>
<td>0.158</td>
<td>0.321</td>
<td>0.154</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>September 12, 2014</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>0.56</td>
<td>3.62</td>
<td>78.4</td>
<td>48.3</td>
<td>4.08</td>
<td>1.24</td>
<td>0.64</td>
<td>0.12</td>
<td>0.27</td>
</tr>
<tr>
<td>MSE</td>
<td>6.10</td>
<td>1.96</td>
<td>40.6</td>
<td>29.8</td>
<td>2.49</td>
<td>0.45</td>
<td>0.34</td>
<td>0.41</td>
<td>0.44</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.011</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LR</td>
<td>2.22</td>
<td>2.63</td>
<td>62.5</td>
<td>37.2</td>
<td>3.17</td>
<td>0.83</td>
<td>0.45</td>
<td>0.22</td>
<td>0.32</td>
</tr>
<tr>
<td>PVLRL</td>
<td>1.65</td>
<td>2.54</td>
<td>55.9</td>
<td>37.3</td>
<td>3.19</td>
<td>0.74</td>
<td>0.47</td>
<td>0.32</td>
<td>0.39</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.695</td>
<td>0.779</td>
<td>0.236</td>
<td>0.975</td>
<td>0.963</td>
<td>0.436</td>
<td>0.726</td>
<td>0.083</td>
<td>0.407</td>
</tr>
<tr>
<td><strong>October 7, 2014</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>0.56</td>
<td>3.14</td>
<td>71.9</td>
<td>44.0</td>
<td>3.73</td>
<td>1.01</td>
<td>0.63</td>
<td>0.25</td>
<td>0.37</td>
</tr>
<tr>
<td>MSE</td>
<td>6.65</td>
<td>1.75</td>
<td>35.8</td>
<td>24.3</td>
<td>2.31</td>
<td>0.39</td>
<td>0.26</td>
<td>0.62</td>
<td>0.64</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.052</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>0.009</td>
<td>0.006</td>
<td>0.001</td>
</tr>
<tr>
<td>LR</td>
<td>1.67</td>
<td>2.32</td>
<td>48.6</td>
<td>32.4</td>
<td>2.72</td>
<td>0.55</td>
<td>0.38</td>
<td>0.45</td>
<td>0.46</td>
</tr>
<tr>
<td>PVLRL</td>
<td>3.33</td>
<td>2.42</td>
<td>49.6</td>
<td>35.8</td>
<td>2.99</td>
<td>0.64</td>
<td>0.43</td>
<td>0.48</td>
<td>0.50</td>
</tr>
<tr>
<td>(P)-value</td>
<td>0.529</td>
<td>0.647</td>
<td>0.917</td>
<td>0.373</td>
<td>0.267</td>
<td>0.499</td>
<td>0.442</td>
<td>0.813</td>
<td>0.703</td>
</tr>
</tbody>
</table>

\(^a\)Percent photon flux (PPF) was not measured on this date
Table 3.2: Effect of basal leaf removal treatments on Noiret vine canopy density and light availability in the fruiting zone during 2015.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PG</th>
<th>LLN</th>
<th>PIC</th>
<th>PIL</th>
<th>OLN</th>
<th>CEL</th>
<th>LEL</th>
<th>CEFA</th>
<th>LEFA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>July 16, 2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81</td>
<td>4.05</td>
<td>89.3</td>
<td>52.9</td>
<td>4.42</td>
<td>1.38</td>
<td>0.75</td>
<td>0.07</td>
<td>0.26</td>
</tr>
<tr>
<td>MSE</td>
<td>5.44</td>
<td>2.52</td>
<td>48.7</td>
<td>38.2</td>
<td>2.95</td>
<td>0.64</td>
<td>0.50</td>
<td>0.40</td>
<td>0.43</td>
</tr>
<tr>
<td>P-value</td>
<td>0.071</td>
<td>0.005</td>
<td>0.038</td>
<td>0.044</td>
<td>0.004</td>
<td>0.012</td>
<td>0.048</td>
<td>0.006</td>
<td>0.012</td>
</tr>
<tr>
<td>LR</td>
<td>5.54</td>
<td>2.48</td>
<td>51.9</td>
<td>37.9</td>
<td>2.93</td>
<td>0.68</td>
<td>0.46</td>
<td>0.36</td>
<td>0.41</td>
</tr>
<tr>
<td>PVLR</td>
<td>3.83</td>
<td>3.43</td>
<td>77.4</td>
<td>48.1</td>
<td>3.82</td>
<td>1.10</td>
<td>0.66</td>
<td>0.19</td>
<td>0.32</td>
</tr>
<tr>
<td>P-value</td>
<td>0.673</td>
<td>0.045</td>
<td>0.075</td>
<td>0.032</td>
<td>0.062</td>
<td>0.044</td>
<td>0.038</td>
<td>0.031</td>
<td>0.038</td>
</tr>
<tr>
<td><strong>September 11, 2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>0.63</td>
<td>3.92</td>
<td>88.7</td>
<td>51.1</td>
<td>4.35</td>
<td>1.33</td>
<td>0.69</td>
<td>0.07</td>
<td>0.26</td>
</tr>
<tr>
<td>MSE</td>
<td>2.75</td>
<td>2.17</td>
<td>40.6</td>
<td>29.2</td>
<td>2.65</td>
<td>0.45</td>
<td>0.34</td>
<td>0.41</td>
<td>0.46</td>
</tr>
<tr>
<td>P-value</td>
<td>0.114</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.008</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LR</td>
<td>1.11</td>
<td>3.47</td>
<td>69.2</td>
<td>47.2</td>
<td>3.84</td>
<td>1.11</td>
<td>0.60</td>
<td>0.24</td>
<td>0.35</td>
</tr>
<tr>
<td>PVLR</td>
<td>8.33</td>
<td>2.26</td>
<td>40.0</td>
<td>36.0</td>
<td>2.75</td>
<td>0.54</td>
<td>0.45</td>
<td>0.41</td>
<td>0.40</td>
</tr>
<tr>
<td>P-value</td>
<td>0.012</td>
<td>0.006</td>
<td>0.040</td>
<td>0.032</td>
<td>0.023</td>
<td>0.008</td>
<td>0.097</td>
<td>0.039</td>
<td>0.207</td>
</tr>
<tr>
<td><strong>October 8, 2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>0.56</td>
<td>3.78</td>
<td>93.1</td>
<td>49.3</td>
<td>4.25</td>
<td>1.58</td>
<td>0.69</td>
<td>0.05</td>
<td>0.28</td>
</tr>
<tr>
<td>MSE</td>
<td>6.11</td>
<td>2.09</td>
<td>39.2</td>
<td>30.7</td>
<td>2.54</td>
<td>0.42</td>
<td>0.34</td>
<td>0.50</td>
<td>0.49</td>
</tr>
<tr>
<td>P-value</td>
<td>0.003</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.005</td>
<td>0.006</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>LR</td>
<td>1.11</td>
<td>2.91</td>
<td>74.5</td>
<td>39.4</td>
<td>3.33</td>
<td>0.97</td>
<td>0.49</td>
<td>0.28</td>
<td>0.41</td>
</tr>
<tr>
<td>PVLR</td>
<td>2.78</td>
<td>2.47</td>
<td>46.0</td>
<td>36.4</td>
<td>2.90</td>
<td>0.55</td>
<td>0.44</td>
<td>0.46</td>
<td>0.48</td>
</tr>
<tr>
<td>P-value</td>
<td>0.374</td>
<td>0.050</td>
<td>0.022</td>
<td>0.393</td>
<td>0.080</td>
<td>0.020</td>
<td>0.524</td>
<td>0.005</td>
<td>0.007</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean values only represent four of the five vineyard replicates for CON measurements on this date.
3.2 Growing Season Temperatures

The cumulative growing degree days (GDDs, base 10°C) from May 1st to harvest during the 2014 and 2015 growing seasons were 1305 and 1419, respectively (Figure 3.1). The grape ripening stage occurred from the end of August (veraison) to October (harvest). During the 2015 growing season, the period from veraison to harvest was characterized by greater heat accumulation (338 GDDs) than 2014 (249 GDDs).

![Graph showing cumulative growing degree days from May to harvest in Geneva, NY during 2014 and 2015.]

**Figure 3.1:** Cumulative growing degree days from 1 May to harvest in Geneva, NY during 2014 and 2015.

May, July, August, and September 2015 had higher GDDs as compared to the same months in 2014 (Figure 3.2). The 2015 growing season showed higher heat accumulation in September, and while both seasons experienced few GDDs in October, 2014 showed higher GDDs during this month than 2015.
Figure 3.2: Comparison of monthly growing degree days from 1 May to harvest during the 2014 and 2015 growing seasons in Geneva, NY.

3.3 Harvest Yield Components and Fruit Chemistries

In 2014, the LR treatment had a lower yield per vine, number of clusters per vine, and berry weight as compared to the PVLR treatment (Table 3.3). Several studies have found that leaf removal conducted between fruit set and veraison does not significantly impact yield components (Bledsoe et al. 1988; Main and Morris 2004; Riccardi 2008; Zoecklein et al. 1992). The significant differences observed for these yield components may have been due to crop losses on the LR vines from the hail storm, which occurred shortly after leaf removal. At the time of this weather event, clusters of LR vines were exposed and susceptible to damage whereas the PVLR vines had dense canopy coverage protecting the fruit. No differences were found in yield parameters between CON and MSE vines in 2014. In 2015, however, the MSE treatment had lower average cluster weight compared to the CON. The reduction in MSE average cluster weight was not caused by a decrease in berry size. It is possible that the MSE vines produced fewer berries per cluster than the
CON vines, but this parameter was not measured. While most studies suggest that cluster weight is not affected by leaf removal conducted after fruit set, Bavaresco et al. (2008) observed reductions in cluster weight as a result of veraison leaf removal in vines of V. vinifera L. cv. Croatina and Malvasia. However, the same treatments applied to Barbera vines growing in the same region did not impact cluster weight; this left authors to suggest that cluster weight may be under genetic control (Bavaresco et al. 2008). No significant differences in the harvest yield parameters were identified between the LR and PVLR treatments in 2015. While an optimum crop load range for Noiret has not been identified in the literature, Bordelon et al. (2008) suggested that crop loads between 8 and 12 were appropriate for the hybrid cultivar Traminette. Therefore, it should be noted that the crop loads for Noiret were below this range for both years studied, indicating that the vines were highly vegetative and likely under cropped.

At harvest in both 2014 and 2015, the TA was significantly lower in the MSE treatment as compared to the CON (Table 3.3). Increased sunlight exposure has been shown to also increase berry surface temperatures; these higher temperatures post-veraison allow for degradation of malic acid through enhanced malate enzyme activity (Percival et al. 1994). There were no significant differences in the other berry chemical parameters (TSS and pH) between treatments for either season.
Table 3.3: Effect of basal leaf removal on Noiret harvest yield components and berry chemistries during 2014 and 2015.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Yield (kg/vine)</th>
<th>Clusters (no./vine)</th>
<th>Cluster weight (g)</th>
<th>Berry weight (g)</th>
<th>Pruning weight (kg/vine)</th>
<th>Crop Load [yield/pruning weight (kg/kg)]</th>
<th>Total soluble solids (°Brix)</th>
<th>pH</th>
<th>Titratable acidity (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2014</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>11.30</td>
<td>142</td>
<td>85.3</td>
<td>1.84</td>
<td>2.04</td>
<td>5.67</td>
<td>17.7</td>
<td>3.27</td>
<td>10.79</td>
</tr>
<tr>
<td>MSE</td>
<td>10.46</td>
<td>147</td>
<td>81.0</td>
<td>1.84</td>
<td>1.83</td>
<td>5.78</td>
<td>18.0</td>
<td>3.28</td>
<td>9.65</td>
</tr>
<tr>
<td>P-value</td>
<td>0.318</td>
<td>0.640</td>
<td>0.313</td>
<td>0.972</td>
<td>0.389</td>
<td>0.862</td>
<td>0.338</td>
<td>0.775</td>
<td>0.009</td>
</tr>
<tr>
<td>LR</td>
<td>10.62</td>
<td>138</td>
<td>85.1</td>
<td>1.70</td>
<td>1.90</td>
<td>6.59</td>
<td>18.2</td>
<td>3.41</td>
<td>8.89</td>
</tr>
<tr>
<td>PVLR</td>
<td>12.54</td>
<td>164</td>
<td>88.2</td>
<td>1.78</td>
<td>2.41</td>
<td>5.70</td>
<td>18.1</td>
<td>3.37</td>
<td>8.68</td>
</tr>
<tr>
<td>P-value</td>
<td>0.050</td>
<td>0.029</td>
<td>0.668</td>
<td>0.014</td>
<td>0.322</td>
<td>0.637</td>
<td>0.501</td>
<td>0.168</td>
<td>0.410</td>
</tr>
<tr>
<td><strong>2015</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>14.97</td>
<td>138</td>
<td>115.3</td>
<td>1.90</td>
<td>3.07</td>
<td>5.09</td>
<td>18.8</td>
<td>3.36</td>
<td>8.53</td>
</tr>
<tr>
<td>MSE</td>
<td>13.55</td>
<td>134</td>
<td>105.2</td>
<td>1.88</td>
<td>3.02</td>
<td>4.53</td>
<td>18.8</td>
<td>3.37</td>
<td>7.69</td>
</tr>
<tr>
<td>P-value</td>
<td>0.409</td>
<td>0.778</td>
<td>0.027</td>
<td>0.652</td>
<td>0.848</td>
<td>0.303</td>
<td>0.790</td>
<td>0.545</td>
<td>0.009</td>
</tr>
<tr>
<td>LR</td>
<td>13.51</td>
<td>125</td>
<td>119.4</td>
<td>1.73</td>
<td>3.22</td>
<td>4.68</td>
<td>18.6</td>
<td>3.45</td>
<td>7.61</td>
</tr>
<tr>
<td>PVLR</td>
<td>15.45</td>
<td>131</td>
<td>122.1</td>
<td>1.79</td>
<td>3.01</td>
<td>5.53</td>
<td>18.7</td>
<td>3.44</td>
<td>7.65</td>
</tr>
<tr>
<td>P-value</td>
<td>0.368</td>
<td>0.687</td>
<td>0.866</td>
<td>0.388</td>
<td>0.652</td>
<td>0.452</td>
<td>0.770</td>
<td>0.631</td>
<td>0.840</td>
</tr>
</tbody>
</table>
3.4 Rotundone

3.4.1 Identification in the Noiret Variety

Rotundone was successfully identified in the Noiret variety as the relative abundances for the ions selectively monitored by mass spectrometry matched those previously reported in the literature (Figure 3.3) (Wood et al. 2008b).

![Figure 3.3: A) Chromatogram of d5-rotundone and rotundone in Noiret grapes, B) mass spectrum of rotundone, corresponding to the peak at retention time 41.554 minutes.](image)

3.4.2 Concentrations in Whole Berries During Growing Season

Rotundone concentrations were not detectable in any samples collected pre-veraison or at 100% veraison for both vintages. In 2014, there were not significant differences in rotundone concentrations between CON and MSE berry samples collected 25 and 57 days after veraison (Figure 3.4A). However, leaf pulling decreased canopy density and increased light availability in the MSE as compared to the CON treatment throughout the ripening stages when rotundone and its precursor are accumulating. Logan (2015)
suggested that the lack of differences in rotundone concentrations arising from sunlight exposure treatments may have been a result of the natural canopies of Shiraz not being dense enough to yield differences in canopy microclimate, specifically berry surface temperatures, as compared to leaf removal treatments. Noiret vines are highly vegetative with dense canopies (Vanden Heuvel et al. 2013). Therefore, Logan’s interpretation is most likely not a reason for the lack of differences in rotundone concentration found in the present study. The timing of leaf removal treatments (LR vs. PVLR) also did not significantly impact rotundone concentrations at harvest in 2014 (Figure 3.4C). Interestingly, the PVLR treatment had significantly ($p < 0.05$) higher rotundone concentrations (0.51 µg/kg) than the LR treatment (0.35 µg/kg) 25 days after veraison. These differences were unexpected as the EPQA data showed that the leaf pulling for the LR and PVLR vines did not result in canopy density or light availability differences at any time throughout the 2014 growing season. The average fruit rotundone concentrations for the 2014 harvest ranged between 3.06 µg/kg (PVLR) and 3.45 µg/kg (CON); these values are relatively high, considering the maximum concentration of rotundone that has been reported in grapes thus far was 5.44 µg/kg in the Vespolina variety (Caputi et al. 2011).

In 2015, rotundone concentration was significantly higher for the MSE treatment as compared to the control midway between veraison and harvest (MSE = 0.22 µg/kg, CON = 0.15 µg/kg; $p < 0.05$) and again at harvest (MSE = 1.98 µg/kg; CON = 1.28 µg/kg; $p < 0.10$) (Figure 3.4B). The increased rotundone concentration associated with the greater light availability to the fruit observed for the MSE vines as compared to the CON opposed expectations based on previous reports. Zhang et al. (2015a) found that a greater extent of natural shading, which corresponds to less sun exposure and lower berry temperatures, resulted in higher rotundone concentrations in Shiraz grapes. There are several possible reasons for the opposing results found in this work. Firstly, defoliation of the vine may elicit a stress response as a signaling mechanism against herbivore attack
(Logan 2015; Martin et al. 2003). This type of response has been reported for sesquiterpenes in other plant species following herbivore damage as an attractant for predators of the feeding herbivores, or as a direct deterrent (Martin et al. 2003). Therefore, the continual pulling of leaves from the MSE vines may have resulted in greater rotundone formation due to recognition of the defoliation as an herbivore attack. Secondly, the changes in sunlight exposure and berry temperatures through leaf removal may have induced more favorable microclimate conditions for the conversion of \( \alpha \)-guaiene to rotundone. The \( VeSTO2 \) enzyme was recently identified as having the ability to biotransform \( \alpha \)-guaiene to rotundone in grapes (Takase et al. 2016). This enzymatic transformation was found to be most favorable at temperatures between 30 and 40°C (Takase et al. 2016). Increased sunlight exposure reaching the fruiting zone via leaf removal has been shown to have the ability to increase berry surface temperatures by as much as 13°C in comparison to shaded clusters (Geffroy et al. 2014). The MSE treatments allowed for greater cluster light interception which likely increased berry temperatures and may have enhanced \( VeSTO2 \) enzyme activity. Beyond enzymatic conversion, aerial oxidation was also proposed as a mechanism for this conversion (Huang et al. 2014). Therefore, increased sun exposure and air temperature induced by leaf removal may advance oxidation as these reactions are favored at higher temperatures (Barter et al. 2015). Again, in 2015, rotundone concentration for PVLR fruit (0.27 \( \mu g/kg \)) was significantly higher (\( p < 0.10 \)) than for the LR fruit (0.22 \( \mu g/kg \)) at the sampling point midway between veraison and harvest with differences in rotundone concentration between treatments disappearing at harvest (Figure 3.4D). This is an interesting observation as the LR and PVLR treatments yielded the same differences in rotundone despite the fact that canopy density and light availability only differed in 2015. It is possible that the development of rotundone, or its precursor \( \alpha \)-guaiene, in these leaf removal treatments is a stress response induced by defoliation.
Several studies have reported that the most influential factor impacting variations in rotundone concentrations is vintage, noting that cooler temperatures, especially between veraison and harvest, result in greater rotundone accumulations in the fruit (Geffroy et al. 2014; Zhang et al. 2015a). The results of this study supported those previously reported as the rotundone concentrations in 2015, the season characterized by warmer temperatures and more cumulative GDDs, were decreased by 63% in the CON, 48% in LR, 40% in PVLR, and 36% in MSE from 2014.

Berry samples taken before and at 100% veraison did not contain detectable levels of rotundone, suggesting that its development occurs between veraison and harvest, as reported in previous studies (Caputi et al. 2011; Geffroy et al. 2014; Logan 2015). September 2015 had higher heat accumulation (273 GDDs) than 2014 (195 GDDs) (Figure 3.2). October 2015 had lower heat accumulation (19 GDDs) than 2014 (55 GDDs). Previous works suggested that the initiation of rotundone development does not occur until three to four weeks after veraison (Herderich et al. 2012; Logan 2015). Based on the timing of veraison observed in this study, rotundone formation would not begin until mid to late September (22 to 29 Sep 2014 and 17 to 24 Sep 2015). The “midway between veraison and harvest” samples were taken shortly after these predicted initiation dates. At this time, the berries contained less than 17% of the rotundone concentration measured at harvest, suggesting that while rotundone was present at this time, the majority of the formation occurred after this sampling point. Interestingly, this time period of increased rotundone development spanned from the last few days of September into late October when GDDs were higher for the 2014 season, which yielded higher concentrations, than 2015.

It is also possible that temperatures between the time of veraison and initiation of rotundone formation impact the rotundone concentration at harvest. Zhang et al. (2015b) found the most significant weather parameter influencing variations in rotundone concentration between vintages was the number of degree hours above 25°C recorded
between veraison and harvest. This parameter was negatively correlated with rotundone concentration at harvest as more degree hours above 25°C resulted in less rotundone formation. In this study, only 50 hours between veraison and harvest were above 25°C in 2014, whereas in 2015, 114 hours were above 25°C between veraison and harvest.

**Figure 3.4:** Impact of cluster sunlight exposure (CON vs. MSE) on rotundone concentration in (A) 2014 and (B) 2015. Impact of the timing of leaf removal (LR vs. PVLR) on rotundone concentration in (C) 2014 and (D) 2015. Berry samples were collected midway between veraison and harvest (25 or 29 days after veraison) and at harvest (57 or 55 days after veraison). Each bar represents the mean of five treatment replicates with error bars expressed as ±SE. A single asterisk (*) indicates a significant difference at p < 0.10 and double asterisks (**) indicate a significant different at p < 0.05.
3.4.3 Wine Chemistry and Rotundone Concentration

No significant treatment differences were observed for pH values measured after malolactic fermentation (MLF), either year (Table 3.4). In 2015, the CON had significantly higher TA as compared to MSE. Vines with dense and highly shaded canopies are known to produce wines with high acidity. Through leaf removal, the increased sun exposure allows for berry surface temperature increases, favoring degradation of malate through enhanced enzyme activity (Percival et al. 1994). These observed differences in the fruit malate concentrations at harvest would suggest that the leaf removal treatment on the MSE vines resulted in increased berry surface temperatures as compared to the CON. In 2014, the alcohol concentration following MLF varied significantly for both treatment comparisons. The CON (12.55%) had higher ethanol concentrations than MSE (11.80%) and the LR treatment (12.10%) had higher alcohol content than PVLR (11.30%). The observed trends between treatments were opposite of those expected based on the vineyard treatments as the MSE and PVLR treatments would most likely enhance ripening through increased sun exposure during the post-veraison stage of development. Furthermore, the tanks were each chaptalized to reach an initial TSS of 21 °Brix. TSS was not measured again directly after sugar additions. Despite efforts to thoroughly mix each bin, measurements would most likely be inaccurate due to poor dissolution as over 1 kg of sugar was added to the cold musts for each treatment. Therefore, the TSS measured the afternoon following yeast inoculation may best reflect the sugar concentrations following adjustments as temperatures were raised through fermentation and the bins had been thoroughly mixed several times. In 2014, the TSS contents the day after additions were 22.60 °Brix for CON, 22.15 °Brix for MSE, 22.25 °Brix for LR, and 20.50 °Brix for PVLR. The treatment differences in alcohol content are likely caused by different sugar concentrations measured for the four treatments, as higher sugar concentrations result in more ethanol formation through yeast metabolism. In 2015, the CON (11.48%) had
significantly lower ethanol content than the MSE treatment (12.50%) while the LR and PVLR treatments were not statistically different. While the observed relationship between CON and MSE is what would be expected based on the differences in sun exposure through canopy manipulation, these vineyard practices are most likely not influencing the results as musts were again chaptalized. During this vintage, the TSS contents the day after additions were 20.45 °Brix for CON, 22.00 °Brix for MSE, 21.75 °Brix for LR, and 21.95 °Brix for PVLR. The variations in initial sugar concentrations closely match those observed for the resulting ethanol concentrations in the wines following MLF. The TSS contents for the LR and PVLR wines were fairly similar following adjustments and this is most likely the reason that significant differences in alcohol content were not observed between these treatments. Variation in sunlight exposure between treatments did not translate to wine color density differences between treatments.

Rotundone concentrations following MLF in 2014 ranged from 183.18 (CON) to 210.62 ng/L (PVLR); in 2015, they ranged from 117.59 (CON) to 223.67 ng/L (PVLR) (Table 3.4). Statistical comparisons could not be made for the CON and MSE treatments as the internal standard was accidentally left out of one of the MSE replicates during preparations. In both 2014 and 2015, rotundone concentration in wine was not affected by the timing of leaf removal (LR vs. PVLR). In 2015, rotundone concentration was 43% lower in the CON wine as compared to the MSE wine. This result confirms the rotundone concentration trend observed for the 2015 CON and MSE fruit at harvest as the CON berries had 36% less rotundone than the MSE berries.

In 2014, the wine post-bottling rotundone concentrations were 186.60 ng/L in CON, 234.24 ng/L in MSE, 248.02 ng/L in LR, and 236.81 ng/L in PVLR. In 2015, the bottled wine concentrations were 147.16 g/L in CON, 227.57 ng/L in MSE, 186.06 ng/L in LR, and 233.50 ng/L in PVLR. Statistical analysis was not performed for the post-bottling wine samples seeing as only one chemical replicate per treatment was available.
Previous studies have reported that approximately 10% of the rotundone originating in the grapes is extracted during fermentation, and only 6% of the initial concentrations were measured in the wines post-bottling (Caputi et al. 2011). In this study, wines from the 2014 vintage showed between 5.32 and 6.84% rotundone extraction following MLF with the 2015 vintage having between 9.20 and 12.17% extraction. Interestingly, these concentrations did not change much between the end of MLF and bottling (one month later) as the 2014 bottled wines had between 5.41 and 7.69% recovery and the 2015 bottled wines had between 11.17 and 12.70% recovery. These concentrations should not increase between MLF and bottling if further biochemical or chemical transformation is not occurring during fermentation. A possible reason for the lower concentrations in the post-MLF samples may be due to scalping of this hydrophobic volatile by plastic centrifuge tubes used for sample storage. The greater extent of rotundone extraction during the 2015 vintage is attributed to the elevated temperatures (28 to 30°C) experienced in the pilot plant processing facility during day 3 of primary fermentation.
Table 3.4: Post-malolactic fermentation chemistry for wines vinified from the Noiret sun exposure (CON vs. MSE) and leaf removal (LR vs. PVLR) treatments. Values represent the average of two fermentation replicates per treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Titratable acidity (g/L)</th>
<th>Alcohol content (%)</th>
<th>Volatile Acidity (g/L)</th>
<th>Color Density (AU)</th>
<th>Rotundone Concentration (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>3.50</td>
<td>7.02</td>
<td>12.55</td>
<td>0.31</td>
<td>-</td>
<td>183.18</td>
</tr>
<tr>
<td>MSE</td>
<td>3.52</td>
<td>6.82</td>
<td>11.80</td>
<td>0.31</td>
<td>-</td>
<td>201.27</td>
</tr>
<tr>
<td>P-value</td>
<td>0.500</td>
<td>0.157</td>
<td>0.042</td>
<td>0.500</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LR</td>
<td>3.62</td>
<td>6.24</td>
<td>12.10</td>
<td>0.31</td>
<td>-</td>
<td>192.92</td>
</tr>
<tr>
<td>PVLR</td>
<td>3.59</td>
<td>6.16</td>
<td>11.30</td>
<td>0.32</td>
<td>-</td>
<td>210.62</td>
</tr>
<tr>
<td>P-value</td>
<td>0.090</td>
<td>0.654</td>
<td>0.040</td>
<td>0.205</td>
<td>-</td>
<td>0.710</td>
</tr>
<tr>
<td>2015</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>3.61</td>
<td>7.10</td>
<td>11.48</td>
<td>0.51</td>
<td>17.21</td>
<td>117.59</td>
</tr>
<tr>
<td>MSE</td>
<td>3.56</td>
<td>6.83</td>
<td>12.50</td>
<td>0.45</td>
<td>16.30</td>
<td>206.78</td>
</tr>
<tr>
<td>P-value</td>
<td>0.070</td>
<td>0.050</td>
<td>0.047</td>
<td>0.293</td>
<td>0.106</td>
<td>0.006</td>
</tr>
<tr>
<td>LR</td>
<td>3.62</td>
<td>6.56</td>
<td>11.90</td>
<td>0.43</td>
<td>17.73</td>
<td>195.52</td>
</tr>
<tr>
<td>PVLR</td>
<td>3.65</td>
<td>6.54</td>
<td>11.85</td>
<td>0.47</td>
<td>18.43</td>
<td>223.67</td>
</tr>
<tr>
<td>P-value</td>
<td>0.268</td>
<td>0.095</td>
<td>0.500</td>
<td>0.393</td>
<td>0.295</td>
<td>0.156</td>
</tr>
</tbody>
</table>

*Parameter not measured at this time for the 2014 vintage

*Only one measurement was acquired for 2014 MSE wine

3.4.4 Correlations Between Berry Rotundone Concentrations, Vineyard Parameters, and Berry Chemistries

Correlation analysis between rotundone concentrations and EPQA parameters, harvest crop yields, pruning weights, and berry chemical components were performed in an effort to determine their impact on rotundone development. In 2014, the pH and TA berry chemical components were correlated with the fruit rotundone concentrations for the samples collected 25 days after veraison (Figure 3.5). The TA was negatively correlated with rotundone while pH was positively correlated with rotundone. These effects are likely related to the extent of ripening of the berries. Previous works suggested an influence of
grape phenology and extent of ripening on rotundone development (Caputi et al. 2011; Zhang et al. 2015b). The practice of leaf removal has been shown to advance ripening and throughout this stage of development, TA decreases and pH increases (Caputi et al. 2011; Creasy and Creasy 2009). Therefore, the observed relationship may be a result of advanced ripening through leaf removal applications, which produces higher rotundone concentrations earlier in the season.

**Figure 3.5:** Relationships between fruit rotundone concentrations and berry A) pH (y = 1.62 x - 4.85, p-value = 0.0063) and B) titratable acidity (y = -0.077 x + 1.20, p-value = 0.0399). The berry samples were collected on 26 Sep 2014.

The rotundone concentrations in the 2014 harvested fruit were positively and linearly correlated with LLN and PIC recorded shortly after veraison (Figure 3.6). This would indicate that a denser canopy (LLN), with more clusters on the interior (PIC) during the time period slightly after veraison, would result in higher rotundone concentrations at harvest.
Figure 3.6: Relationships between fruit rotundone concentrations at harvest (28 Oct 2014) and EPQA metrics A) leaf layer number \( y = 0.32 x + 2.34, \text{p-value} = 0.0333 \) and B) percent interior clusters \( y = 0.014 x + 2.37, \text{p-value} = 0.0201 \) recorded on 12 Sep 2014.

Furthermore, rotundone concentrations in the 2014 harvested fruit were negatively correlated with yield per vine and crop load (Figure 3.7). Interpretation of these results would indicate that vines with lower fruit yields at harvest and less vigor produced fruit with higher rotundone concentrations. However, rotundone concentration at harvest was not significantly correlated with the number of clusters per vine, cluster weight berry weight, or pruning weight. Scarlett et al. (2014) found that rotundone concentrations were not solely driven by vine vigor and suggested that the land underlying the vineyard partially influences the accumulation of rotundone in grapes. Logan (2015) and Geffory et al. (2014) also found that crop load reductions through crop thinning treatments did not significantly impact rotundone concentrations in the fruit at harvest or the wine, respectively.
**Figure 3.7:** Relationship between fruit rotundone concentrations at harvest (28 Oct 2014) and A) yield per vine \( (y = -0.11x + 4.41, \text{p-value} = 0.0128) \) and B) crop load \( (y = -0.11x + 3.87, \text{p-value} = 0.0166) \).

In 2015, the rotundone concentrations in the mid-ripening berry samples were found to be negatively correlated with PIC and positively correlated with CEFA at the EPQA time point slightly after veraison (Figure 3.8). This would indicate that greater light interception by clusters (greater CEFA) and fewer clusters in the interior of the canopy (lower PIC) through leaf removal practices results in greater rotundone concentrations midway between veraison and harvest.
Figure 3.8: Relationships between fruit rotundone concentrations midway between veraison and harvest (25 Sep 2015) and EPQA metrics A) percent interior clusters \((y = -0.0013 \ x + 0.29, \ \text{p-value} = 0.0265)\) and B) cluster exposure flux availability \((y = 0.21 \ x + 0.16, \ \text{p-value} = 0.0138)\) recorded on 11 Sep 2015.

Additionally, the rotundone concentrations at harvest in 2015 also exhibited a positive relationship with CEFA (Figure 3.9). This would indicate that greater amounts of cluster light interception shortly after veraison resulted in increased amounts of rotundone at harvest.

Figure 3.9: Relationship between fruit rotundone concentrations on at harvest (21 Oct 2015 and cluster exposure flux availability \((y = 1.26 \ x + 1.33, \ \text{p-value} = 0.0462)\) on 11 Sep 2015.
In looking at these correlations as a whole, it seems that light interception during the first half of the ripening stage of development (veraison to initiation of rotundone formation) is important in influencing the rotundone concentrations in the fruit midway between veraison and harvest and at harvest. No significant linear correlations were found between rotundone concentration at harvest and EPQA canopy density and cluster light availability parameters recorded in the late stages of ripening when rotundone is accumulating. Parameters influencing rotundone and the general trends for these correlations were not consistent between the 2014 and 2015 seasons; therefore, the relationships between rotundone concentration and EPQA parameters, harvest crop yields, pruning weights, and berry chemical components warrant further investigation.

3.5 Sensory

3.5.1 Discrimination of Relative Rotundone Concentrations in Wine

During the training portion of the sensory trial, a triangle test was completed by 65 panelists with the intent to identify individuals who were able to distinguish between wines with different black pepper aroma intensities. Twenty-nine panelists (44% of the panelists) correctly identified the odd sample, which equaled the minimum number of correct responses required to establish a confidence interval of 95%. Many of the panelists who correctly completed the task described one wine as having a strong or intense pepper aroma while the other wine exhibited a softer, lighter, or muted overall aroma with less pepper and more fruit character. The results of the triangle test were not used as a means for eliminating panelists as a group of at least 60 individuals was necessary for the sensory evaluation. The selection of wines for the discrimination analysis was considered not ideal as the rotundone concentrations were fairly similar between the two samples (2014 LR = 248.02 ng/L; 2014 MSE = 234.24 ng/L). Wines with greater differences in rotundone concentration would have been chosen had the MDGC-MS analysis been completed prior to the sensory analysis, but time constraints did not allow for this in the present work.
3.5.2 Correlation Between Black Pepper Intensity Rating and Rotundone Concentration in Noiret Wines

Previous works found that perceived black pepper aroma intensity is well correlated with rotundone concentration in wine (Geffroy et al. 2016; Wood et al. 2008b). This relationship was also explored in the present study by plotting mean black pepper aroma intensity rating versus rotundone concentration for the Noiret wines (Figure 3.10). Vintage and treatment effects were determined to be not significant, and therefore, both vintages were combined for this analysis. Black pepper intensity and rotundone concentration in wine showed a significant linear correlation ($R^2 = 0.6082$, p-value = 0.0225), confirming the findings of earlier reports.

![Graph showing correlation between Black Pepper Intensity Rating and Rotundone Concentration](image)

$r = 0.7906$

**Figure 3.10:** Mean black pepper intensity ratings versus rotundone concentration for 2014 and 2015 Noiret wines. Regression equation: $y = 0.090 \times x + 32.07$, p-value = 0.0225.
Chapter 4

Conclusions and Future Work

4.1 Conclusions

This study investigated the effects of cluster sunlight exposure and timing of leaf removal on rotundone development in Noiret grapes and wine. Rotundone was successfully identified in the Noiret variety (an interspecific hybrid of Vitis), confirming the hypothesis that this compound contributes to the spicy, black pepper aroma which is characteristic to wines of this variety. Furthermore, this work was the first to identify the presence of rotundone in a hybrid variety grown within the northeastern United States climate.

Rotundone was found to accumulate in the late stages of ripening (ca. 4 weeks before harvest), supporting the previously reported developmental trends. Comparison of rotundone concentration differences between the respective sunlight exposure and timing of leaf removal treatments did not support the hypothesis that rotundone concentrations would be reduced when viticultural practices allow for increased sunlight exposure to reach the fruiting zone during the ripening stage of development. For both vintages, post-veraison leaf removal (PVLR) fruit had significantly higher rotundone concentrations than pre-veraison leaf removal (LR) fruit sampled midway between veraison and harvest, but the rotundone concentrations were no longer significantly different in fruit sampled at the
time of harvest. It is possible that the observed higher rotundone concentration in PVLR fruit mid-ripening was caused by a stress response induced by defoliation of the vine. While the maintained 100% sun exposure (MSE) and control (CON) fruit did not exhibit differences in rotundone concentrations during 2014, the MSE fruit contained higher rotundone concentrations than the CON in samples collected both midway between veraison and harvest and at harvest in 2015. These higher observed rotundone concentrations with continuous leaf pulling may be a result of prolonged vine stress via defoliation or a product of enhanced rotundone formation with increased sunlight exposure through enzymatic or non-enzymatic oxidation mechanisms. Furthermore, vintage variation has been found to be one of the most important factors influencing rotundone development with cooler temperatures throughout ripening resulting in greater accumulations. Harvest fruit in the 2014 season, which was characterized by cooler temperatures and less cumulative growing degree days, had higher rotundone concentrations than fruit harvested in 2015. Linear regression analysis between rotundone concentrations and enhanced point quadrat analysis (EPQA) parameters suggested that light interception during the first half of the ripening stage of development may influence the rotundone concentrations in the fruit midway between harvest and at harvest.

The plot of black pepper aroma intensity ratings versus rotundone concentrations in the wines resulted in a significant linear relationship, confirming the hypothesis that black pepper intensity ratings were positively correlated with rotundone concentrations in the wines vinified from the viticultural treatments.

4.2 Future Work

Following the identification of rotundone in the Noiret variety, future studies should investigate the presence of this compound in other United States grown wine grape varieties. Furthermore, studies of rotundone developmental dynamics in various rotundone-containing cultivars grown within the cool northeastern United States climates
would allow for comparison of the environmental and cultural factors influencing rotundone development in this region with those found in Australia, France, Italy, and New Zealand. In addition, it would be interesting to conduct a survey of non-hybrid wine grape species other than *Vitis vinifera* (i.e. *V. riparia, V. labrusca, V. rupestris*) in order to determine whether the presence of rotundone is a *V. vinifera* specific trait.

In this study, the timing of leaf removal treatments (LR and PVLR) could not be compared to the CON as these vines were not rooted in the same way. Another similar study should be employed with all treatments implemented on Noiset vines of the same rootstock type. Comparison of all four treatments may allow for a better understanding of the mechanism driving the observed trends. Furthermore, it would be interesting to not only monitor rotundone, but also investigate the impact of these vineyard management techniques on berry surface temperatures, \( \alpha \)-guaiene development, and *VuSTO2* activity. Analysis of berry surface temperatures would first allow for determination of whether the cluster sunlight exposure treatments are altering the temperature of the fruit and if so, to what extent. This may allow for a better understanding of whether the observed trends are a being influenced by light interception or the changes in fruit temperature, which have found to result from increased sunlight exposure. Monitoring \( \alpha \)-guaiene developmental dynamics and *VuSTO2* enzyme activity in response to the viticultural treatments may provide further understanding regarding the mechanism of rotundone formation.

It would also be beneficial to conduct a separate study to determine whether the herbivore attack stress mechanism is playing a role in the observed differences in rotundone concentration as a result of leaf removal. This investigation could be carried out by assessing the effects of timing and duration of leaf removal from the apex of the vine shoots. Removal of leaves from sections of the shoots that are not contributing to the canopy coverage in the fruiting zone would allow for fruit temperatures to be maintained by the natural foliage while also potentially eliciting an herbivore attack stress response.
Furthermore, the regression analysis performed in this study suggested that light interception during the first half of the ripening stage of development (veraison to initiation of rotundone accumulation) may be an important factor influencing rotundone concentrations in the fruit at mid-ripening and harvest. Therefore, it would also be interesting to study the impact of timing and duration of leaf removal post-veraison on rotundone development, possibly through maintained sun exposure during the first four weeks of ripening as compared to leaf removal during the late stages of ripening when rotundone is accumulating.

While descriptive analysis successfully allowed for the identification of a significant linear correlation between black pepper aroma intensity and rotundone concentration in wine, further study investigating the impact of timing and duration of cluster sunlight exposure on the perceived black pepper aroma intensities in the wines is warranted. Vineyard management techniques are known to affect the development of several grape-derived aroma compounds in addition to rotundone. The wines vinified in this work were anecdotally described by wine experts as having distinctly different overall aroma profiles. Therefore, it would be interesting to evaluate additional aroma descriptors, beyond black pepper, in the wines produced from the vineyard treatments to better understand factors influencing the perception of this black pepper attribute. In addition to a more comprehensive descriptive analysis study, a preference test of the same wines conducted with a consumer panel could be used to determine which vineyard treatment yields the wine that is most liked by consumers. Combining the data from these two sensory tests may allow for growers to better understand the impact of these management practices on wine quality, potentially allowing them to choose the best technique to achieve the most desirable wine aroma profile.
Bibliography


Slegers, A., P. Angers, É. Ouellet, T. Truchon, and K. Pedneault. 2015. Volatile compounds from grape skin, juice and wine from five interspecific hybrid grape cultivars grown in Québec (Canada) for wine production. Molecules 20:10980-11016.


