THE SIGNIFICANCE OF VITICULTURAL MANAGEMENT AND VINIFICATION DECISIONS ON WINE QUALITY PARAMETERS - ELEMENTAL SULFUR RESIDUES AND C_{13} NORISOPRENOID PRECURSORS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Misha Taylor Kwasniewski

January 2013

THE SIGNIFICANCE OF VITICULTURAL MANAGEMENT AND VINIFICATION DECISIONS ON WINE QUALITY PARAMETERS - ELEMENTAL SULFUR RESIDUES AND C_{13} NORISOPRENOID PRECURSORS

Misha Taylor Kwasniewski, Ph. D.

Cornell University 2013

A series of studies were undertaken to better characterize influences of viticultural and vinification decisions on wine quality parameters, including those that affect aging potential.

Elemental sulfur (S^0) is commonly used to control powdery mildew, a ubiquitous disease of grapevines. While beneficial over alternative control products in many respects, late season S^0 applications increase the potential for undesirable "reduced" aroma development in wine produced from treated fruit, as residual S^0 concentrations >1-10 μ/g increase H_2S evolution during fermentation. However, the persistence of S^0 in the vineyard and through vinification is poorly understood, partially owing to the limitations of previous methods for S^0 analysis in media that contain other forms of sulfur. A simple, economical technique was developed to quantify S^0 residues on grapes in the vineyard and throughout vinification. The technique is based upon complete conversion of S^0 to H_2S and the subsequent capture and quantification of that gas using commercially available detection tubes. This method was then utilized to analyze grape and must samples from 3 years of field trials, in which the variable factors were S^0 formulation, dose, and application timing relative to harvest. Additionally, a series of investigations was undertaken to better understand the impact that increased H_2S production during fermentation has on final wine chemistry. While formulation and application rate affected S^0

residue concentration and persistence for some treatments, timing between final treatment and harvest consistently had an effect on final residue concentrations. Cessation of S^0 application 35 days or more prior to harvest resulted in S^0 levels below 10µg/g for all treatments. For some treatments, harvest S^0 residues below 10µg/g were observed even when spraying was ceased as late as 22 days before harvest.

In wine produced from the S^0 field trials in which elevated H_2S production was observed, there was also increased incidence of H_2S formation in finished wines post bottling, even though no H_2S was present at bottling. The H_2S that re-emerged 3-weeks and 6-months post bottling correlated well with H_2S produced during fermentation. H_2S produced during fermentation also correlated well with a "latent" pool of H_2S that was releasable by treating the wine with a reducing agent.

In a separate experiment, the effects of the timing of light exposure on grape derived volatiles was explored. Fruiting zone leaf removal is a common practice to increase light exposure, which has been shown to reduce disease pressure and positively influence ripening. The compound TDN (1,1,6-trimethyl 1,2-dihydronaphthalene) has been linked to the classic "petrol-like" aroma found in some Riesling wines. Higher concentrations of TDN precursors in grapes has been associated with increased cluster sun exposure. A field trial was undertaken in which fruit zone leaf removal was applied at three different timings. Concentrations of carotenoids and bound and free TDN and other volatiles were quantified in harvested berries and in wines. Leaf removal at 33-days post-berry set significantly increased zeaxanthin in Riesling grapes mid-season, total TDN and vitispirane in the juice of mature Riesling grapes, and free and total TDN in finished wine, while earlier or later leaf removal had no effect.

BIOGRAPHICAL SKETCH

The author grew-up in Portland, NY, a town along Lake Erie, which becomes enveloped by the aroma of Concord grapes every Fall. He was exposed early to hard work farming life, opting to leave after high school to find less risky work as a bike messenger in Philadelphia and free-lance photographer. He worked for local newspapers during high-school and was a staff photographer at the Cortland Standard and the Elmira Star Gazette in Central NY. He first gained an interest in fermentation starting with home brewing in Philadelphia while also developing interest in horticulture through making the most out of an inhospitable roof garden. Due to an ever-growing interest in the technical details of winemaking and horticulture he decided to return to school to formally pursue the subject. In Fall of 2007, he enrolled in the newly formed undergraduate degree program in Viticulture and Enology at Cornell University. During this time as an undergraduate at Cornell he was afforded many opportunities to obtain research experience. Having seen the value that a strong relationship between grape and wine producers, research universities and extension have on the industry as a whole he decided to pursue a graduate degree in Food Science at Cornell focusing on enology and flavor chemistry research. Following the completion of his degree he will take a position as an Assistant Research Professor and Enology Program Leader at the University of Missouri at Columbia.

To Chris, my partner in the struggle

ACKNOWLEDGMENTS

This work is the result of a great deal of work and support by too many people to be able to properly acknowledge all of their efforts. The help from my Committee, the Food Science and New York State Agricultural Experiment Station in Geneva Staff and Faculty, Grower Cooperators, Extension and my fellow Viticulture and Enology classmates has been essential.

Dr. Gavin Sacks' infectious interest in understanding "why" showed me that it was possible to be earnestly excited about research and simply learning something new, a skill that not every academic researcher possess. Dr. Wayne Wilcox's faith in my ability to pursue my graduate studies and beyond has been an important motivator along this journey. Dr. Justine Vanden Heuvel's support as an undergraduate thesis advisor was integral to my initial grad-school success and led to a chapter in this document. Relating to that research I would also like to thank the efforts of Dr. Bruce Pan, who not only taught me a great deal about analytical chemistry but also prevented me from losing fingers while attempting my first chemical synthesis. A thank you to Dr. Terry Acree for demonstrating such enthusiasm, which encouraged me to see the rewards of an academic career despite the many challenges. Kathleen Arninck taught me the fundamentals of conducting quality lab work for wine analysis and has always made time to talk. Luann Preston-Wilsey and Pam Raes Cornell Vinification and Brewing Lab have been instrumental in supporting my research wine production and minimizing the associated stress of that hectic time during harvest season. Many thanks to Duane Riegel for taking care of the tedious business of my complicated spray treatments. Thanks to Dr. James Meyers , Dr. Trent Preszler, and Dr. Justin Scheiner for keeping me on my toes with thoughtful discussions.

Without the support and Deb and Hal Kwasniewski I would have never had the confidence to get here. Despite my sometimes abysmal performance in school they were always supportive, working toward finding alternative ways for me to learn and pursue my interests. With long hours working at my Mother's greenhouses teaching me more about horticulture than I ever learned at Cornell (though I didn't

know it at the time). My parents instilled in me the belief that you can be considered successful no matter what you do, as long as you do something with passion.

Lastly, I cannot overstate the role my partner Dr. Christine Costello has played in getting me to the point that I could start, let alone complete this work. Since we first met years ago, discussing physics over a beer at a bar in Philadelphia, she has provided me with the motivation to succeed and a model to emulate.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1
INTRODUCTION

Overview

A wine's flavor is the sum of every decision and uncontrollable influence that the grapevines, fruit and wine undergo until being enjoyed[1]. While many decisions are made during the production of wine with the intent of improving quality, these decisions set off a cascade of events that can influence flavor long after they are made[2]. A wine's "life" can be divided into three major periods, the vineyard, the winery and the bottle. The chemistry at each of these periods decides what reactions are possible in the future, whether abiotic or microbially mediated. At each of these stages decisions made by the producer can influence flavor character, although that influence diminishes greatly as the wine's life progresses[3]. Once quality has been compromised little can be done to restore it, and it is therefore necessary to be able to predict how decisions made throughout the process will ultimately affect a wine.

The difficulty in understanding the system of wine comes from the complexity and interrelated nature of the many factors affecting quality. The major chemical constituents of wine are relatively simple and include water, acid (malic and tartaric) and ethanol. Acids are accumulated early in ripening and degraded toward harvest as sugars accumulate in the grape[4]. The sugars are then converted to ethanol and carbon dioxide (CO_2) during fermentation[5]. Concentrations of acids and sugars are influenced by many factors including: grape variety, climate, sun exposure, crop-load, disease and directly though water content in the fruit[6]. These factors can result in grapes with acid and sugar concentrations varying among producers by two- to threefold. Additional differences can occur during fermentation, as sugar-to-ethanol conversion rates are influenced by yeast strain and malic acid can be converted to lactic when specific bacteria are used[5, 7]. Because acids and sugars are relatively easy to quantify with minimal lab facilities, they are routinely measured by wineries to facilitate educated decision-making during harvest and processing, e.g., the addition of sugar prior to fermentation to increase final alcohol concentrations.

Wine is, however, much more than ethanol, acid and water. Many critical flavor compounds or their precursors exist at ng/kg or lower concentrations, and are challenging to measure in even well-equipped wineries[2]. In many cases, this leaves winemakers to rely on their experience and intuition for decision making, which puts them at a disadvantage when encountering novel situations like new grape varieties or unexpected weather conditions. By better characterizing the system that begins at bud-break and continues until the wine is in the glass, research serves to better equip winemakers and growers so they can adapt to unknown situations.

This dissertation research aimed to characterize effects of specific viticultural practices on grape composition and eventual wine flavor. The primary focus was understanding the fate of elemental sulfur on grapes and wines. Elemental sulfur application in the vineyard has been linked to "reduced" character in finished wine, which can be described as burnt tire- or rotten vegetable-like. Elemental sulfur (S^0) is known to increase H_2S, a contributor to reductive aromas, during fermentation. However, little research had been done to characterize the persistence of S^0 in the vineyard and through vinification, in part due to an absence of simple and affordable methodology for S^0 quantification in media containing additional forms of sulfur. Thus, initial work focused on developing a method for S^0 quantification appropriate both for research and commercial settings (Chapter 2). This method was then used to characterize S^0 spray residue persistence during ripening and through vinification over three years of field trials (2009-2011) in Finger Lakes vineyards (Chapter 3). Despite negligible levels of H_2S observed at bottling, measureable concentrations of H_2S was produced in many of the fermentations, which correlated to increased H_2S in the wines after storage. Chapter 4 outlines efforts to characterize possible sources for the re-emerging H_2S during bottle aging and outlines initial efforts to develop a method for predicting a wine's propensity to develop unwanted reductive aromas.

Chapter 5 explores a different compound while addressing the same basic question, i.e., how do various factors during grape production and wine making influence the final flavor profile. A C_{13}-norisoprenoid, l,l,6-trimethyl-1,2-dihydronaphthalene (TDN), is associated with "kerosene" or "petrol" aromas in some Riesling wines[8]. This character is generally considered a fault in young wines but an

element of quality in aged Riesling wines. Although a positive correlation between fruit sun exposure and eventual wine TDN concentrations is well established, the effect of exposure timing on TDN potential has not been previously investigated [9, 10].

Wine Sulfur Aroma

Volatile Sulfur Compounds (VSCs) are among the most odor active in wine, with some VSCs having aroma thresholds at the ng/L to µg/L levels[11]. Some of these compounds, such as 3-mercaptohexanol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP), are essential for the varietal aroma of wines such as Sauvignon Blanc [12]. The first broad class of these compounds exist in grapes as non-odorous precursors in the form of S-cysteine or S-glutathione conjugates, and are released during fermentation [13]. Though most recognizable in Sauvignon Blanc, they also exist above threshold levels in other wines [14]. The other broad class of VSCs is primarily derived from yeast activities such as amino acid synthesis [15], though other factors such as glutathione breakdown or abiotic chemical reduction of elemental sulfur may also contribute to their formation [16]. These fermentation-derived VSCs include H_2S, methyl mercaptan and ethyl mercaptan as well as disulfides formed by these compounds when oxygen is present. Such compounds are generally regarded as faults when above their recognition threshold, as they evoke such descriptors as rotten egg, onion, burnt rubber or garlic. Wines with this type of character are described sensorially as being "reduced". Many of these malodorous compounds contain a thiol group and bind readily to transition metals, a trait winemakers frequently exploit in their removal by copper fining. However when copper is added prophylactically at bottling it may actually increase free H_2S is the wine [17].

Despite adoption by many winemakers of appropriate practices to limit the potential for production of VSC compounds and to mediate their impact when present, many wines are nevertheless found to be commercially unacceptable due to reduced aromas [18]. This may be linked to the concurrent adoption, in recent years, of more reductive wine making techniques and screwcaps in place of cork

closures [17]. Both of these factors will cause the wine's chemistry to shift to and/or remain in a more reductive state, favoring the preservation and formation of VSC's. While there is evidence that VSC's such as H_2S can increase during anaerobic storage, there remains a poor understanding of the wine components that serve as precursors for VSC's during storage[19]. Some research efforts have focused on thiols and disulfides. It is currently possible to test for the presence of thiols in wine at odor-active concentrations through the addition of copper sulfate, which subsequently binds these compounds. Similarly disulfides are tested for with the addition of ascorbic and copper, though this test's sensitivity and mechanism have not been characterized [20]. Disulfides have been suggested as a possible latent pool of VSCs, e.g. dimethyldisulfide could release methyl mercaptan during aging[21], although this would not explain the evolution of H_2S. Furthermore, oxidation of a mercaptan-spiked wine resulted in the loss of mercaptans but did not increase concentrations of the corresponding disulfide[22].

An alternative explanation for the emergence of thiols after bottling may be due to the fact that thiols are nucleophiles and have a propensity to react with electrophiles [23]. This is important both to the fate of desirable VSCs such as 3MH in Sauvignon Blanc, as well as undesirable VSCs such as H_2S. One possibility involves thiols reacting with oxidized wine phenolic compounds (quinones) prior to bottling, with the reaction reversing to release VSCs during ageing [24]. While it has been shown that the reaction between quinones and thiols can take place [23-25], the ability for this reaction to reverse has not been demonstrated under wine conditions. It has previously been hypothesized that "free" H_2S produced during fermentation becomes bound to unknown compounds in a reaction that is reversible during bottle aging [26]. However, whereas several potential sinks for H_2S have been characterized, the reversibility of these reactions has not.

Impact of elemental sulfur residues on VSC formation and wine aroma

Elemental sulfur (S^0) is an effective and inexpensive control for the most economically devastating vineyard pathogen, grapevine powdery mildew (PM) [27]. However, elevated levels of S^0 residues at harvest

can increase H_2S production during fermentation and undesirable "reduced" aroma in the finished wine [16, 26, 28, 29]. There is currently insufficient knowledge related to the persistence of S^0 in the vineyard and during vinification for growers and winemakers to properly assess the risks of late season application of S^0. Without a better understanding of these relationships, economic losses are likely to occur due to either the production of faulted wine resulting from S^0 application too close to harvest or from overreliance on expensive alternatives to S^0, which entail the additional risk of eventual failure caused by pathogen resistance development.

S^0 residues on grapes have long been known to result in H_2S production during fermentation [28-31], and several studies have attempted to define maximum S^0 residues to avoid excess H_2S production. However, precise recommendations on limiting S^0 sprays have been complicated by the fact that even in the absence of S^0 content, H_2S is produced during fermentation as a byproduct of amino acid synthesis during normal yeast (*Saccharomyces cerevisiae*) metabolism[15], and is affected by factors such as juice nutrition [32], turbidity[29], yeast strain[29], and fermentation temperature [26] as well as S^0 content [29, 33]. There is good agreement that S^0 concentrations $\geq$10µg/g in a fermentation can increase H_2S formation. Reports on the effect of S^0 additions between 1-10 µg/g have been mixed. In one experiment, H_2S production doubled when micronized S^0 was added at a rate of 2 µg/g to fermentations utilizing a high H_2S producing yeast strain [34]. Other groups have reported increased H_2S production with S^0 as low as 1 µg/g[35, 36]. However, Thomas et al. (1993) reported that additions of dusting S^0 to must at a concentration of 3.4 µg/g did not consistently cause an increase in H_2S production, and that addition of 1.7 µg/g S^0 actually caused a decrease in H_2S production with some yeast strains. This disagreement may be due in part to the use of different particle sizes of S^0among the different studies, as smaller particle size has been shown to be more effective at increasing H_2S production; however, other factors such as turbidity and yeast strain have also been shown to affect H_2S output among fermentations. Higher ethanol content, must temperature, turbidity and high H_2S-producing yeast strains have all been linked to a greater impact of S^0 on H_2S production[26, 33]. There is a large degree of variation witnessed in studies when defined growth media or sterile filtered juice have not been used. This variation makes it difficult to understand the lower limit at

which S^0 residue impacts H_2S production, and studies using commercial vinification practices to ferment grape juice have shown large variations in H_2S production within treatments[31, 37]. For instance, Acree et al. reported that H_2S production among replicates of the same treatments, using aliqouts of the same juice and yeast strain, ranged from 32-660 ng/g following S^0 additions and from 14-46 ng/g without addition[31]. To summarize, S^0 concentrations >10 μg/g in must are expected to produce increased H_2S, and musts with 1-10 μg/g of S^0 may be at risk for increased H_2S production.

Though S^0 residues can increase H_2S production during fermentation this does not necessarily equate with increased H_2S in the finished wine. Due to the high reactivity and volatility of H_2S, it has been demonstrated that even in fermentations with high H_2S, little if any will exist at bottling[31, 34]. During fermentation most H_2S will exit through the airlock with the CO_2 also produced during fermentation. However, the H_2S remaining in the wine, though greatly diminished in volume, has the potential to further react with electrophiles in the wine and produce numerous new compounds, including aroma-active compounds such as methanethiol [25, 35]. In the presence of an electron donor such as copper (a common addition for H_2S removal), additional compounds can be formed [23, 38]. With the low aroma detection threshold of H_2S and H_2S reaction products, there is still potential for deleterious effects on wine quality should even a small proportion of H_2S-based aroma compounds remain. It should be noted that while H_2S resulting from yeast metabolism is primarily produced early in the fermentation, a second peak of H_2S production has been observed at the end of fermentation if S^0 residue is present, and in some cases this second peak persists after all fermentables have been consumed[26, 39], likely because there is less opportunity to be entrained and removed by CO_2 as fermentation concludes.

Factors affecting S residues on grapes

While a number of studies have characterized the effects of S^0 on H_2S production, only a very limited number have quantified S^0 residues on grapes as the result of different viticultural or pre-fermentation practices, and these are somewhat contradictory. Thomas et al. found that applications of

10-17 kg/ha of dusting S^0 applied until veraison resulted in S^0 concentrations on grapes <14 μg/g one day after application, <4 μg/g two weeks after, and 1-3.4 μg/g at harvest [49]. In contrast, others have found residue levels as high as 8 μg/g at harvest when applications ceased 7-weeks prior to harvest[36].

Vinification decisions are another area potentially important to S^0 persistence into the must. It has been previously demonstrated that clarification can greatly reduce S^0 levels in white wine must, leading to a reduction in H_2S production [33, 36]. However, the impacts of decisions such as whole cluster pressing or the length of skin contact are not well understood. As a result, it is not obvious how the standard range of white winemaking practices will affect S^0 residues.

S^0 Analysis

Studies of the persistence of S^0 application would be facilitated by convenient methods for trace level (sub part per million) detection. This is necessary both for research regarding the persistence of S^0 in the vineyard and through vinification, but also to offer producers a way to assess risk under their individual conditions. Classic wet chemical approaches first convert S^0 to a more readily detectable product, e.g., $S_2O_3^{2-}$ [40] or $Fe(SCN)_6^{3-}$ [41], which are then measured by polarography or spectrophotometry, respectively. An alternate wet chemical approach is to convert the S^0 to H_2S through the addition of Cr^{2+} [42] or by treatment with Cu^0 following acetone extraction [43]. The evolved sulfide can then be measured by several approaches, including a sulfide-selective electrode [44] or colorimetric methods [45]. These approaches can suffer from poor selectivity and require handling and disposal of transition metals or organic solvents, including CCl_4, tetrachloroethylene, CS_2 and toluene. Newer methods rely on direct measurement of S^0 by HPLC [46], GC-MS [47] or ion chromatography [48]. Such methods usually involve a pre-concentration/extraction step with organic solvents prior to analysis, making them inappropriate for informal laboratories or field settings. Furthermore, newer methods based on chromatography require trained operators and specialized equipment, and thus are impractical for modestly-equipped labs.

A number of problems with the previous methods used by Wenzel et al. and Thomas et al. to quantify S^0 residue on grapes necessitated the development of a new assay [33,49]. Both previous assays quantified S^0 residue levels that were washable from the surface of the berry, a point of potentially significant loss due to the limited solubility of S^0 in the solvents used, either petroleum ether[36], or an aqueous surfactant (Tween) [49]. Neither extraction method was validated. Also, although the S^0 quantification by HPLC was validated by Wenzel et. al. [39], the ICP method used by Thomas et al. (1993) to quantify S^0 was not. This not only draws into the question the validity of their results, but also makes their methods unacceptable for use without further validation. While it may be possible to modify either of these methods for research, pending further validation, both nevertheless use equipment for S^0 quantification that would not be available to most in the wine industry. As S^0 residues are potentially variable depending on site, year, canopy density and other factors specific to an individual growing site, the best option to allow the industry to make educated decisions about their S^0 applications was to develop an assay appropriate for both research and industry.

Carotenoid Derived Aromas

The C_{13}-norisoprenoids are one of several classes of grape-derived odor-active compounds associated with wine aroma quality [2]. While trace levels of free C_{13}-norisoprenoids are detectable in juice, the majority of C_{13}-norisoprenoids in wine appear to derive from precursors, including non-volatile C_{13}-norisoprenoids glycosides derived from carotenoid cleavage [50], and can be released during winemaking or storage by enzymatic and non-enzymatic mechanisms [51]. The best-studied C_{13}-norisoprenoid in wine and grapes is arguably TDN (l,l,6-trimethyl-1,2-dihydronaphthalene), which is associated with “kerosene” or “petrol” aromas and has an orthonasal sensory threshold of 20 µg/L in wine [8]. TDN has been detected in several varietal wines, but its presence is most closely associated with the aroma of bottle-aged Riesling [52]. While TDN concentrations around sensory threshold are generally acceptable to consumers, excessive levels are considered undesirable, especially in young Riesling [52].

Free TDN in Riesling juice is generally below detection threshold, but TDN concentrations in excess of 200 µg/L in Riesling wine are reported to occur following prolonged storage [8, 53]. TDN precursors, e.g. C_{13}-norisoprenoid glycosides, have been reported in grapes, and the concentration of TDN in a finished wine is proportional to the concentration of acid-releasable TDN precursors in must [2, 52]. Warmer growing conditions and greater cluster exposure to sunlight are associated with higher TDN concentrations in finished wines, due to a larger concentration of precursors in the juice [52]. Conversely, lower TDN concentrations in wine are associated with fruit shaded either through direct means like canopy management [10] or indirectly through increased vine fertilization[54] or irrigation[55] that lead to increased vine canopy. A similar decrease in the concentration of several other volatile C_{13}-norisoprenoid precursors has been observed in shaded clusters, including vitispirane and the actinidols [56]. One possible exception to this trend is β-damascenone, which has been implicated in enhancing fruity aromas in wines. Some authors have reported an increase in β-damascenone in response to cluster shading [10, 52], while others have reported either no change or a decrease in shaded grapes [9, 57].

Because of the clear link between TDN precursor production and cluster light exposure, and assuming that lower TDN concentrations are desirable, a superficially obvious solution to reducing the TDN potential of Riesling or other winegrapes would be to minimize cluster exposure. However, increasing berry sun exposure is often desirable for reducing disease pressure [58-60], decreasing titratable acidity, and potentially for increasing production of other desirable compounds like monoterpenes [61]. Therefore, it is advantageous to identify canopy management practices that will produce desirable outcomes independent of C_{13}-norisoprenoid concentrations, especially TDN. A better understanding of the key period(s) during the growing season in which cluster sun exposure increases C_{13}-norisoprenoid precursors could assist winegrape growers in making appropriate canopy management decisions for targeting specific wine flavor profiles.

The (bio-)chemical mechanisms underlying C_{13}-norisoprenoid precursor formation in grapes have been subject to considerable study [2, 62]. TDN and other C_{13}-norisoprenoids show structural similarities to carotenoids, and there is strong evidence that C_{13}-norisoprenoid precursors in mature grapes are derived

via oxidative degradation of carotenoids [63]. The major carotenoids in grapes, β-carotene and lutein, begin to decrease at or just prior to veraison [64]. C_{13}-norisoprenoid precursor formation commences within 1-2 weeks after veraison and may reach a maximum within 30-days post-veraison, although some studies report a late spike in concentration near maturity [52, 63]. Grape C_{13}-norisoprenoids were originally proposed to be formed by abiotic carotenoid degradation, e.g., TDN can be formed from lutein under acidic conditions [65]. Alternatively, a family of carotenoid cleavage dioxygenase (CCD) enzymes has been implicated in production of plant apocarotenoids, e.g. C_{13}-norisoprenoids [66], and a CCD capable of producing C_{13}-norisoprenoids from lutein and zeaxanthin (*VvCCD1*) was recently cloned from grapes [50]. Expression of *VvCCD1* increases at veraison, although a 1-2 week lag is reported to occur between increased transcript expression and a significant increase in glycosylated C_{13}-norisoprenoids. Following enzymatic or non-enzymatic biogenesis, part of the pool of C_{13}-norisoprenoids is proposed to undergo *in vivo* glycosylation, potentially after further transformations (e.g. hydration, oxidation) within the grape berry [62, 67] (Figure 1). Grape-derived C_{13}-norisoprenoid glycosides can be hydrolyzed during fermentation and storage, and both native and glycoside derived C_{13}-norisoprenoid aglycones can be further transformed enzymatically or non-enzymatically to odor active forms, e.g. TDN and β-damascenone [68, 69].

Carotenoids are expressed in photosynthetically active tissues of plants as part of Photosystem II (PSII). The major carotenoid species in grapes (β-carotene, lutein) act as light harvesting antennae pigments, while other oxygenated carotenoid species (e.g. neoxanthin, zeaxanthin) participate in photo-protection of the plant via the xanthophyll cycle [70]. Total carotenoid concentrations are believed to be primarily developmentally regulated [70], but environmental factors such as cluster light exposure also influence concentrations [71-73]. Since pre-veraison berries are photosynthetically active, higher concentrations of carotenoids, and thus higher substrate availability, are one potential explanation for higher concentrations of C_{13}-norisoprenoid precursors in sun-exposed grapes [71]. However, cluster exposure does not consistently yield higher concentrations of carotenoids pre-veraison [74]. A second explanation is that post-veraison cluster exposure may accelerate carotenoid degradation, possibly by increasing *VvCCD1* expression [75], although the effect of sun exposure on increasing carotenoid

degradation rates has also been disputed [55]. A third potential explanation is that sun exposure results in conversion of epoxyxanthophylls (e.g. violaxanthin) to de-epoxidized xanthophylls (e.g. zeaxanthin). Since the putative starting point for the precursors of TDN, vitispirane, and related compounds may be de-epoxidized xanthophylls [62], sun exposure may alter the proportion of de-epoxidized vs. epoxidixed forms of xanthophylls, and these different substrates could yield different C_{13} norisoprenoid precursors post-veraison [63]. Berries exposed to sun pre-veraison are reported to have a higher proportion of de-epoxidized xanthophylls [73] than shaded berries, but a clear correlation between a specific carotenoid or carotenoids in pre-veraison grapes and eventual concentrations of TDN or other C_{13}-norisoprenoids in mature fruit has not been conclusively demonstrated.

In summary, increased cluster exposure may increase concentrations of TDN precursors and related compounds through one or more mechanisms, including greater accumulation of carotenoids, faster carotenoid degradation, or increased availability of specific carotenoid substrates. This lack of understanding of the relationship between C_{13}-norisoprenoids and light is inadequate for designing viticultural management strategies to avoid TDN precursor production while ensuring an open canopy to reduce disease and improve fruit composition. Many reports have studied the relationship of TDN precursor concentrations to cluster light exposure, yet none have considered manipulating the timing of the cluster exposure treatment to produce the optimum effect.

References

1. Jackson, R.S. *Wine science: principles and applications.* Academic Press: 2008;

2. Rapp, A. Volatile flavour of wine: Correlation between instrumental analysis and sensory perception. *Nahrung-Food* **1998,** *42,* 351-363.

3. Schreier, P.; Jennings, W.G. Flavor composition of wines: a review. *Critical Reviews in Food Science & Nutrition* **1979,** *12,* 59-111.

4. Kliewer, W.M. Influence of environment on metabolism of organic acids and carbohydrates in Vitis vinifera. I. Temperature. *Plant Physiol.* **1964,** *39,* 869.

5. Benítez, T.; Del Castillo, L.; Aguilera, A.; Conde, J.; Cerdáolmedo, E. Selection of wine yeasts for growth and fermentation in the presence of ethanol and sucrose. *Appl. Environ. Microbiol.* **1983,** *45,* 1429-1436.

6. Jackson, D.; Lombard, P. Environmental and management practices affecting grape composition and wine quality-a review. *Am. J. Enol. Vitic.* **1993,** *44,* 409-430.

7. Davis, C.; Wibowo, D.; Eschenbruch, R.; Lee, T.; Fleet, G. Practical implications of malolactic fermentation: a review. *Am. J. Enol. Vitic.* **1985,** *36,* 290-301.

8. Simpson, R.F. 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene: An important contributor to the bottle aged bouquet of wine. *Chem. Ind.* **1978,** *1,* 37.

9. Marais, J.; Wyk, C.J.; Rapp, A. Effect of sunlight and shade on norisoprenoid levels in maturing Weisser Riesling and Chenin blanc grapes and Weisser Riesling wines. *South African Journal for Enology and Viticulture* **1992,** *13,* 23-32.

10. Lee, S.; Seo, M.; Riu, M.; Cotta, J.P.; Block, D.E.; Dokoozlian, N.K.; Ebeler, S.E. Vine microclimate and norisoprenoid concentration in cabernet sauvignon grapes and wines. *Am. J. Enol. Vitic.* **2007,** *58,* 291-301.

11. Landaud, S.; Helinck, S.; Bonnarme, P. Formation of volatile sulfur compounds and metabolism of methionine and other sulfur compounds in fermented food. *Appl. Microbiol. Biotechnol.* **2008,** *77,* 1191-1205.

12. Tominaga, T.; Murat, M.L.; Dubourdieu, D. Development of a method for analyzing the volatile thiols involved in the characteristic aroma of wines made from Vitis vinifera L. cv. Sauvignon blanc. *J. Agric. Food Chem.* **1998,** *46,* 1044-1048.

13. Tominaga, T.; des Gachons, C.P.; Dubourdieu, D. A New Type of Flavor Precursors in Vitis v inifera L. cv. Sauvignon Blanc: S-Cysteine Conjugates. *J. Agric. Food Chem.* **1998,** *46,* 5215-5219.

14. Tominaga, T.; Baltenweck-Guyot, R.; Des Gachons, C.P.; Dubourdieu, D. Contribution of volatile thiols to the aromas of white wines made from several Vitis vinifera grape varieties. *Am. J. Enol. Vitic.* **2000,** *51,* 178-181.

15. Jiranek, V.; Langridge, P.; Henschke, P. Regulation of hydrogen sulfide liberation in wine-producing Saccharomyces cerevisiae strains by assimilable nitrogen. *Appl. Environ. Microbiol.* **1995,** *61,* 461.

16. Acree, T.E.; Sonoff, E.P.; Splittstoesser, D.F. Effect of yeast strain and type of sulfur compound on hydrogen sulfide production. *Am. J. Enol. Vitic.* **1972,** *23,* 6.

17. Ugliano, M.; Kwiatkowski, M.; Vidal, S.; Capone, D.; Siebert, T.; Dieval, J.B.; Aagaard, O.; Waters, E.J. Evolution of 3-mercaptohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. *J. Agric. Food Chem.* **2011,**

18. Siebert, T.E.; Solomon, M.R.; Pollnitz, A.P.; Jeffery, D.W. Selective determination of volatile sulfur compounds in wine by gas chromatography with sulfur chemiluminescence detection. *J. Agric. Food Chem.* **2010,**

19. Lopes, P.; Silva, M.A.; Pons, A.; Tominaga, T.; Lavigne, V.; Saucier, C.; Darriet, P.; Teissedre, P.L.; Dubourdieu, D. Impact of oxygen dissolved at bottling and transmitted through closures on the composition and sensory properties of a Sauvignon Blanc wine during bottle storage. *J. Agric. Food Chem.* **2009,** *57,* 10261-10270.

20. Zoecklein, B.W.; Fugelsang, K.C.; Gump, B.H.; Nury, F.S. *Wine analysis and production.* Chapman & Hall New York: 1995;

21. Limmer, A. The chemistry of post-bottling sulfides in wine. *Chemistry in New Zealand* **2005,** *69,* 2.

22. Majcenovic, A.B.; Schneider, R.; Lepoutre, J.P.; Lempereur, V.; Baumes, R. Synthesis and stable isotope dilution assay of ethanethiol and diethyl disulfide in wine using solid phase microextraction. Effect of aging on their levels in wine. *J. Agric. Food Chem.* **2002,** *50,* 6653-6658.

23. Waterhouse, A.L. Recent Observations in Wine Oxidation. *MACROWINE 2010 Proceedings - 3rd International Symposium on Macromolecules and Secondary Metabolites of Grapevine and Wines* **2010,**

24. Cheynier, V.F.; Trousdale, E.K.; Singleton, V.L.; Salgues, M.J.; Wylde, R. Characterization of 2-S-glutathionyl caftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986,** *34,* 217-221.

25. Nikolantonaki, M.; Chichuc, I.; Teissedre, P.L.; Darriet, P. Reactivity of volatile thiols with polyphenols in a wine-model medium: Impact of oxygen, iron, and sulfur dioxide. *Anal. Chim. Acta* **2010,** *660,* 102-109.

26. Schutz, M.; Kunkee, R.E. Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast. *Am. J. Enol. Vitic.* **1977,** *28,* 137.

27. Gadoury, D.M.; Cadle-Davidson, L.; Wilcox, W.F.; Dry, I.B.; Seem, R.C.; Milgroom, M.G. Grapevine powdery mildew (Erysiphe necator): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. *Molecular plant pathology* **2011,** *13,* 1-16.

28. Sluiter, E. The production of hydrogen sulphide by animal tissues. *Biochem. J.* **1930,** *24,* 549.

29. Rankine, B.C. Nature, origin and prevention of hydrogen sulphide aroma in wines. *J. Sci. Food Agric.* **1963,** *14,* 79-91.

30. Nessler, J. *Die bereitung, pflege und untersuchung des weines.* E. Ulmer: 1898;

31. Acree, T.; Sonoff, E.P.; Splittstoesser, D. Effect of yeast strain and type of sulfur compound on hydrogen sulfide production. *Am. J. Enol. Vitic.* **1972,** *23,* 6.

32. Ugliano, M.; Fedrizzi, B.; Siebert, T.; Travis, B.; Magno, F.; Versini, G.; Henschke, P.A. Effect of nitrogen supplementation and Saccharomyces species on hydrogen sulfide and other volatile sulfur compounds in Shiraz fermentation and wine. *J. Agric. Food Chem.* **2009,** *57,* 4948-4955.

33. Wenzel, K.; Dittrich, H. Zur Beeinflussung der Schwefelwasserstoff-Bildung der Hefe durch Trub, Stickstoffgehalt, molekularen Schwefel und Kupfer bei der Vergärung von Traubenmost. *Wein-Wissenschaft* **1978,** *33,* 200-213.

34. Park, S.K. Development of a method to measure hydrogen sulfide in wine fermentation. *Journal of microbiology and biotechnology* **2008,** *18,* 1550-1554.

35. Thoukis, G.; Stern, L.A. A Review and some Studies of the Effect of Sulfur on the Formation of Off-Odors in Wine. *Am. J. Enol. Vitic.* **1962,** *13,* 133-140.

36. Wenzel, K.; Dittrich, H.; Seyffardt, H.; Bohnert, J. Schwefelrückstände auf Trauben und im Most und ihr Einfluss auf die H 2 S-Bildung. *Wein-Wissenschaft* **1980,** *35,* 414-420.

37. Thomas, C.S.; Boulton, R.B.; Silacci, M.W.; Gubler, W.D. The effect of elemental sulfur, yeast strain, and fermentation medium on hydrogen sulfide production during fermentation. *Am. J. Enol. Vitic.* **1993,** *44,* 211.

38. Nedjma, M.; Hoffmann, N. Hydrogen sulfide reactivity with thiols in the presence of copper (II) in hydroalcoholic solutions or cognac brandies: Formation of symmetrical and unsymmetrical dialkyl trisulfides. *J. Agric. Food Chem.* **1996,** *44,* 3935-3938.

39. Wenzel, K. Die HPLC-Bestimmung von elementarem Schwefel in Lebensmitteln. *Zeitschrift für Lebensmitteluntersuchung und-Forschung A* **1980,** *170,* 5-6.

40. Luther III, G.W.; Giblin, A.E.; Varsolona, R. Polarographic analysis of sulfur species in marine porewaters. *Limnol. Oceanogr.* **1985,** 727-736.

41. Bartlett, J.K.; Skoog, D.A. Colorimetric determination of elemental sulfur in hydrocarbons. *Anal. Chem.* **1954,** *26,* 1008-1011.

42. Fossing, H.; Jørgensen, B.B. Measurement of bacterial sulfate reduction in sediments: evaluation of a single-step chromium reduction method. *Biogeochemistry* **1989,** *8,* 205-222.

43. Hardman, A.F.; Barbehenn, H.E. Determination of free sulfur in rubber. *Industrial & Engineering Chemistry Analytical Edition* **1935,** *7,* 103-104.

44. Berner, R.A. Distribution and diagenesis of sulfur in some sediments from the Gulf of California. *Mar. Geol.* **1964,** *1,* 117-140.

45. Cline, J.D. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **1969,** *14,* 454-458.

46. Henshaw, P.F.; Bewtra, J.K.; Biswas, N. Extraction of elemental sulfur from an aqueous suspension for analysis by high-performance liquid chromatography. *Anal. Chem.* **1997,** *69,* 3119-3123.

47. Chen, Y.W.; Joly, H.A.; Belzile, N. Determination of elemental sulfur in environmental samples by gas chromatography-mass spectrometry. *Chem. Geol.* **1997,** *137,* 195-200.

48. Fabbri, D.; Locatelli, C.; Tarabusi, S. A new procedure, based on combustion to sulphate and ion chromatography for the analysis of elemental sulphur in sediments. *Chromatographia* **2000,** *53,* 119-121.

49. Thomas, C.S.; Gubler, W.D.; Silacci, M.W.; Miller, R. Changes in elemental sulfur residues on Pinot noir and Cabernet Sauvignon grape berries during the growing season. *Am. J. Enol. Vitic.* **1993,** *44,* 205.

50. Mathieu, S.; Terrier, N.; Procureur, J.; Bigey, F.; Gunata, Z. A Carotenoid Cleavage Dioxygenase from Vitis vinifera L.: functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. *J. Exp. Bot.* **2005,** *56,* 2721-2731.

51. Jesús Ibarz, M.; Ferreira, V.; Hernández-Orte, P.; Loscos, N.; Cacho, J. Optimization and evaluation of a procedure for the gas chromatographic–mass spectrometric analysis of the aromas generated by fast acid hydrolysis of flavor precursors extracted from grapes. *Journal of Chromatography A* **2006,** *1116,* 217-229.

52. Marais, J.; Van Wyk, C.; Rapp, A. Effect of storage time, temperature and region on the levels of 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene and other volatiles, and on quality of Weisser Riesling wines. *South African Journal for Enology and Viticulture* **1992,** *13,* 33-44.

53. Winterhalter, P.; Sefton, M.A.; Williams, P.J. Volatile C-13-Norisoprenoid Compounds in Riesling Wine are Generated from Multiple Precursors. *Am. J. Enol. Vitic.* **1990,** *41,* 277-283.

54. Linsenmeier, A.W.; Lohnertz, O. Changes in norisoprenoid levels with long-term nitrogen fertilisation in different vintages of Vitis vinifera var. riesling wines. *South African Journal of Enology and Viticulture* **2007,** *28,* 17-24.

55. Bindon, K.A.; Dry, P.R.; Loveys, B.R. Influence of plant water status on the production of C-13-norisoprenoid precursors in Vitis vinifera L. Cv. Cabernet Sauvignon grape berries. *J. Agric. Food Chem.* **2007,** *55,* 4493-4500.

56. Marais, J. The significance of 1, 1, 6-trimethyl-1, 2-dihydronaphthalene in the production of high quality Riesling wines, In *ACS Symposium Series 802, Carotenoid-derived aroma compounds,* Winterhalter, P. and Rouseff, R.L., Eds.; Washington, DC; American Chemical Society: 2002; Vol.802 pp. 273.

57. Ristic, R.; Downey, M.O.; Iland, P.G.; Bindon, K.; Francis, I.L.; Herderich, M.; Robinson, S.P. Exclusion of sunlight from Shiraz grapes alters wine colour, tannin and sensory properties. *Aust J Grape Wine Res* **2007,** *13,* 53.

58. Zoecklein, B.W.; Wolf, T.K.; Duncan, N.W.; Judge, J.M.; Cook, M.K. Effects of fruit zone leaf removal on yield, fruit composition, and fruit rot incidence of Chardonnay and White Riesling (Vitis vinifera L.) grapes. *Am. J. Enol. Vitic.* **1992,** *43,* 139.

59. Austin, C.N.; Grove, G.G.; Meyers, J.M.; Wilcox, W.F. Powdery mildew severity as a function of canopy density: Associated impacts on sunlight penetration and spray coverage. *Am. J. Enol. Vitic.* **2011,** *62,* 23-31.

60. Austin, C.N.; Wilcox, W.F. Effects of fruit-zone leaf removal, training systems, and irrigation on the development of grapevine powdery mildew. *Am. J. Enol. Vitic.* **2011,** *62,* 193-198.

61. Reynolds, A.G.; Wardle, D.A.; Naylor, A.P. Impact of training system, vine spacing, and basal leaf removal on Riesling. Vine performance, berry composition, canopy microclimate, and vineyard labor requirements. *Am. J. Enol. Vitic.* **1996,** *47,* 63-76.

62. Mendes-Pinto, M.M. Carotenoid breakdown products the—norisoprenoids—in wine aroma. *Arch. Biochem. Biophys.* **2009,** *483,* 236-245.

63. Baumes, R.; Wirth, J.; Bureau, S.; Gunata, Y.; Razungles, A. Biogeneration of C-13-norisoprenoid compounds: experiments supportive for an apo-carotenoid pathway in grapevines. *Anal. Chim. Acta* **2002,** *458,* 3-14.

64. Razungles, A.J.; Babic, I.; Sapis, J.C.; Bayonove, C.L. Particular behavior of epoxy xanthophylls during veraison and maturation of grape. *J. Agric. Food Chem.* **1996,** *44,* 3821-3825.

65. Marais, J. 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene (TDN): a possible degradation product of lutein and beta-carotene. *South African Journal of Enology and Viticulture* **1992,** *13,* 52-55.

66. Auldridge, M.E.; McCarty, D.R.; Klee, H.J. Plant carotenoid cleavage oxygenases and their apocarotenoid products. *Curr. Opin. Plant Biol.* **2006,** *9,* 315-321.

67. Mathieu, S.; Wirth, J.; Sauvage, F.X.; Lepoutre, J.P.; Baumes, R.; Gunata, Z. Biotransformation of C 13-norisoprenoids and monoterpenes by a cell suspension culture of cv. Gamay (Vitis vinifera). *Plant Cell, Tissue and Organ Culture* **2009,** *97,* 203-213.

68. Meyers J., Sacks G., and Vanden Heuvel J. Naturally occurring spatial variability in canopy biomass impacts flavor and aroma compounds in riesling. *Am. J. Enol. Vitic.* **2009,** *60,* 394a-395a.

69. Winterhalter, P.; Schreier, P. C13-Norisoprenoid glycosides in plant tissues: An overview on their occurrence, composition and role as flavour precursors. *Flavour Fragrance J.* **1994,** *9,*

70. Hirschberg, J. Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* **2001,** *4,* 210-218.

71. Marais, J.; Van Wyk, C.J.; Rapp, A. Carotenoid levels in maturing grapes as affected by climatic regions, sunlight and shade. *S.Afr.J.Enol.Vitic* **1991,** *12,* 64-69.

72. Bureau SM; Razungles AJ; Baumes RL; Bayonove CL Effect of vine or bunch shading on the carotenoid composition in Vitis vinifera L. berries. I. Syrah grapes. *Wein-Wissenschaft Viticultural and Enological Sciences* **1999,** *53,* 53 (2) 64-71.

73. Düring, H.; Davtyan, A. Developmental changes of primary processes of photosynthesis in sun- and shade-adapted berries of two grapevine cultivars. *Vitis* **2002,** *41,* 63-67.

74. Oliveira, C.; Ferreira, A.C.; Costa, P.; Guerra, J.; de Pinho, P.G. Effect of some viticultural parameters on the grape carotenoid profile. *J. Agric. Food Chem.* **2004,** *52,* 4178-4184.

75. Razungles, A.J.; Baumes, R.L.; Dufour, C.; Sznaper, C.N.; Bayonove, C.L. Effect of sun exposure on carotenoids and C-13-norisoprenoid glycosides in Syrah berries (Vitis vinifera L.). *Sci. Aliments* **1998,** *18,* 361-373.

Chapter 2

QUANTIFICATION OF ELEMENTAL SULFUR IN GRAPE SAMPLES

Abstract

Rapid, inexpensive, and convenient methods for quantifying elemental sulfur (S^0) with low or sub-µg g−1 limits of detection would be useful for a range of applications where S^0 can act as a precursor for noxious off-aromas, e.g., S^0 in pesticide residues on winegrapes or as a contaminant in drywall. However, existing quantification methods rely on toxic reagents, expensive and cumbersome equipment, or demonstrate poor selectivity. We have developed and optimized an inexpensive, rapid method (~15 min per sample) for quantifying S^0 in complex matrices. Following dispersion of the sample in PEG-400 and buffering, S^0 is quantitatively reduced to H_2S in situ by dithiothreitol and simultaneously quantified by commercially available colorimetric H2S detection tubes. By employing multiple tubes, the method demonstrated linearity from 0.03 to 100 µg S^0 g^{-1} for a 5 g sample (R^2 = 0.994, mean CV = 6.4%), and the methodological detection limit was 0.01 µg S^0 g^{-1}. Interferences from sulfite or sulfate were not observed. Mean recovery of an S^0 containing sulfur fungicide in grape macerate was 84.7% with a mean CV of 10.4%. Mean recovery of S^0 in a colloidal sulfur preparation from a drywall matrix was 106.6% with a mean CV of 6.9%. Comparable methodological detection limits, sensitivity, and recoveries were achieved in grape juice, grape macerate and with 1 g drywall samples, indicating that the methodology should be robust across a range of complex matrices.

Introduction

Convenient methods for trace level (sub part per million) measurements of elemental sulfur (S^0) are of relevance to the agricultural, geological, and environmental sciences. For example, S^0 is commonly used as a fungicide for powdery mildew control on grapes and other agricultural crops, but S^0 residues can be converted to noxious H_2S during alcoholic fermentations, e.g., during production of wine, beer and sake [29]. In the case of grapes, despite the well-understood and deleterious effects of sulfur spray residues on wine quality [16, 29], the measurement of S^0 is not routinely performed prior to harvest or fermentation, likely due to the lack of a methodology appropriate for the modest laboratory facilities found at most commercial wineries. Similarly, microbial reduction of S^0 in gypsum drywall has been suggested as a source of H_2S in homes afflicted by "Chinese drywall syndrome" [74], and on-site measurements of S^0 content would be valuable. Beyond these field applications, laboratory analyses of S^0 in complex matrices are important to a range of fields, e.g., in studies of geochemistry [75], wastewater treatment [76], petrochemicals [77], and forensic analysis [78], and developing inexpensive and rapid means for quantifying S^0 in complex samples is of general interest.

Existing methods for S^0 measurement are reviewed in detail by Kamyshny et al. and others [48, 79] and are outlined in Table 2.1. Classic wet chemical approaches first convert S^0 to a more readily detectable product, e.g., $S_2O_3^{2-}$ [41] or $Fe(SCN)_6^{3-}$ [42], which are then measured by polarography or spectrophotometry, respectively. An alternate wet chemical approach is to convert the S^0 to H_2S through the addition of Cr^{2+} [43] or by treatment with Cu^0 following acetone extraction [44]. The evolved sulfide can then be measured by several approaches, including a sulfide-selective electrode [45] or colorimetric methods [46]. These approaches can suffer from poor selectivity and require handling and disposal of transition metals or organic solvents, including CCl_4, tetrachloroethylene, CS_2 and toluene. Newer methods rely on direct measurement of S^0 by HPLC [47], GC-MS [48] or ion chromatography [49]. Such methods usually involve a pre-concentration/extraction step with organic solvents prior to analysis, making them inappropriate for informal laboratories, field settings, or household use. Furthermore, newer

methods based on chromatography required trained operators and specialized equipment, and thus are impractical for modestly equipped labs.

Table 2.1. Overview of Current Elemental Sulfur Detection Methods Compared to Proposed Method

Method of quantification	Reagent/Sample preparation	Measured species	Citation	Sample matrix	Sample size (g)	LLOQ ($\mu g\ g^{-1}$)
Detection Tube (4LT)	DTT reduction	H_2S	This paper	aqueous (Grape)	5	0.02
Detection Tube (4LL)	DTT reduction	H_2S	This paper	aqueous (Grape)	5	0.25
Electrode	Hydrazine reduction	S^-	*80*	cosmetics	0.5	2
Spectro-photometry	Chromium reduction	ZnS	*81*	sediment	1	5
Gravimetric	Conversion to H_2S by Cu mesh in acetone	$BaSO_4$	*82*	sediment	5	30
HPLC	Direct	S	*83*	sediment	10	4
HPLC	Direct	S	*84*	aqueous	5	50
GC-MS	Direct	S	*48*	sediment	1	0.1
Ion Chromatography	Combustion	sulfate	*49*	sediment	1	27
Colorimetry	Fe^{3+} and CN-	$FE(SCN)_6$	*75*	sediment	1	0.8
Pulse-Polarography	Na_2SO_4	thiosulfate	*85*	aqueous	2	≈0.2

We describe the development, optimization, and validation of a safe, inexpensive, and novel method for S^0 quantification, in which S^0 is converted quantitatively to H_2S by a mild thiol reducing agent and simultaneously detected by commercial sulfide detection tubes. The approach was validated in three complex matrices for which convenient S^0 measurement should have immediate utility: grape juice, grape macerate, and drywall.

Experimental

Chemicals

Commercially purchased chemicals - Cysteine, glutathione, mercaptoethanol, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), sodium sulfide, sodium hydroxide and polyethylene glycol 400 (PEG 400) were all purchased at ≥99% purity (Fischer Scientific). Elemental sulfur (S^0-E, orthorhombic) at the highest available purity and colloidal sulfur (S^0-CS), 80% w/w S^0 were purchased from Sigma-Aldrich. Microthiol® (S^0-MS), a water-dispersable, wettable powder, 80% w/w S^0 fungicide formulation from Cerexagri-Nisso (King of Prussia, PA) was used; Alka-Seltzer tablets (Bayer Healthcare, Morristown, NJ) and simethicone tablets (Quality Choice Extra-Strength Gas Relief, Novi, MI) were purchased locally. Ultra high purity nitrogen gas was used (Airgas, Ithaca, NY). Distilled de-ionized water was used for all experiments. Two commercial suppliers of H_2S detection tubes were utilized: Sulfur Stick™ Cat No. 99-001 and 99-005 (Sang Il Int'l Corp, South Korea) and Gastec 4LT, 4LL and 4H (Nextteq, Tampa, FL). The detection tubes rely on a colorimetric reaction within the tube between evolved H_2S and a metal salt, either mercury chloride (Gastec 4LT detection tube) or lead acetate (all other detection tubes) adhered to a proprietary, inert matrix. The length of the tube darkened is linearly proportional to the quantity of H_2S evolved, where a change of 0.5mm (the smallest detectable change), is equivalent to 0.005 µg, 0.1 µg and 5 µg of S^0 on 4LT, 4LL and 4H tubes respectively.

Preparation of H_2S Calibration Standards - A S^{2-} stock solution was prepared using a Silver/Sulfide electrode (Orion Cat No. 9616BNWP) according to the electrode manufacturer's specifications. Briefly, 100 g of Na_2S*9H_20 was dissolved in 100 g water, and diluted 1:100 in deaerated 1M NaOH. The true concentration of S^{2-} was established by titration against a 0.1 M $Pb(ClO_4)_2$ solution (Orion Cat No. 948206). The stock solution was kept refrigerated and retested weekly. Calibration standards were prepared in duplicate over appropriate ranges for each sulfide detection tube by addition of stock solution to deaerated buffer. The buffer composition is described below. Standards were deaerated initially by N_2 gas sparging and in later experiments by addition of Alka-Seltzer tablets. Dissolved O_2 was <0.1 µg mL^{-1} determined by a Hach LDO handheld dissolved oxygen meter (Loveland, CO).

Preparation of S^0 calibration standards- The methodology for preparing S^0-E standards in PEG 400 was adopted from a previous report [86]. Reagent grade S^0-E was dissolved in PEG 400 while stirring in a 100°C water bath to prepare a 3.2 mg mL^{-1} stock solution, which was serially diluted in PEG 400 to make 0.032 and 0.32 mg mL^{-1} solutions. These solutions were then used to create calibration standards at the rates described below. The solutions were held at 80°C prior to use to prevent precipitation. Stock solutions of S^0-MS and S^0-CS were prepared by suspending the formulation in water at S^0 concentrations (w/w) equivalent to those for S^0-E and were stirred prior to use.

2.2 Optimization of Methodology 2.2.1 H_2S measurement apparatus- Two apparatus for measuring H_2S in solution were compared. Apparatus #1 was adopted from Park [34] Figure 2.1. Briefly, the headspace of a 500 mL Erlenmeyer flask was sparged with N_2 gas through a H_2S detection tube while the sample is vigorously stirred with a stir bar. Apparatus #2 was adapted from a protocol described by a commercial vendor of H_2S detection consumables [87]. A 120 ml glass flask containing the liquid sample was sparged by successive addition of two Alka-Seltzer tablets 5 min apart, which generated CO_2. The effluent was directed to an H_2S detection tube connected to the reaction flask via a short piece of silicone tubing. Contamination due to foaming was avoided by use of a 5 mL volumetric glass pipette between

the reaction flask and detection tube (Figure 2.2) and by addition of silicone oil or a crushed simethicone-containing tablet to the sample.

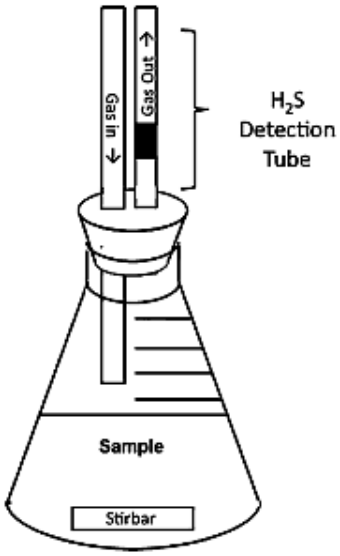

Figure 2.1. Schematic of original Apparatus #1 for S^0 measurement, adopted from Park 2008.

H_2S formed following S^0 reduction is purged from solution by a gas stream and detected by reaction with a metal salt containing sulfur detection tube.

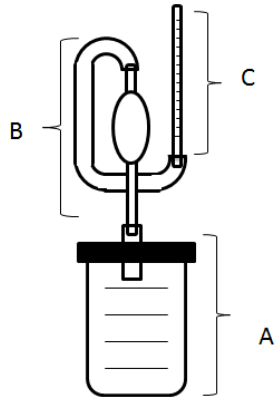

Figure 2.2. Schematic of Apparatus #2 and Summary of Assay.

Following dispersion in PEG 400 (1:4 ratio), the sample is combined with 80 mL H_2O in a 120 mL glass screw-top flask (A) fitted with a 5 mL glass pipette as a condenser (B). The sample is buffered and deaerated by addition of an Alka-Seltzer tablet, and DTT is added along with another Alka-Seltzer tablet. The evolved H_2S is detected by a commercial sulfide detection tube (C).

Evaluation of Reducing Agents- The effectiveness of several reducing agents in reducing S^0 to S^{2-} was tested: glutathione, cysteine, mercaptoethanol, DTT, and TCEP. S^0-E dissolved in PEG 400 (3.2 mg mL^{-1}) was added to 200 mL of 5 mg mL^{-1} citric acid to yield a final concentration of 0.25 µg mL^{-1} S^0. The sample was adjusted to pH 11 with 1M NaOH, a reducing agent (20 mM) was added, and the sample was incubated at 50°C for 30 minutes. Then, the pH was adjusted to 3.0 using 1M phosphoric acid and H_2S was quantified using Apparatus #1, described above. The yield of H_2S from S^0-E was determined by comparing the response achieved from S^0-E standards to H_2S standards.

Optimization of pH for concurrent formation/quantification of H_2S- Buffered solutions of 5 g L^{-1} citric acid were prepared over a range of pH values from 3.0 to 10.0 by adjustment with 1M NaOH. S^0-E dissolved in PEG 400 (3.2 mg ml^{-1}) was added to 200 ml of 5 mg ml^{-1} citric acid to yield a final concentration of 0.25 µg ml^{-1} S^0. DTT was then added to yield a final concentration of 20 mM, the sample incubated at 50°C for 30 min., and H_2S quantified by Apparatus #1.

Optimization of sample pre-treatment with PEG 400- H_2S recoveries were determined from a S^0-MS calibration standard (2 µg mL^{-1} as S^0). First, different ratios of PEG 400 to sample volume (1:9, 1:4, 1:2, 1:1, 2:1 3:1, 4:1, 6:1, 9:1) were prepared, heated to 80 °C for 10 min., and then analyzed for S^0. Then, using the optimal PEG 400-to-sample ratio so determined (1:4), the effect of temperature on S^0 recovery was evaluated at 22, 30, 40, 55, 65, 70, 80, 90, and 100 °C. Finally, the effect of extraction time was evaluated (2, 3, 4, 5, 7.5, 15, 30 min.) at the optimum temperature of 80 °C. Analyses were done in duplicate, using Apparatus #2, the previously optimized S^0 protocol, and Gastec 4LL detection tubes.

Optimized S^0 Analysis Methodology Used to Test Recovery, Detection Limits, and Interferences- A 1 to 5 g aqueous sample or 1 g dry sample (e.g., drywall) is added to PEG 400 at a 1:4 ratio in a 120 mL flask and heated in a 80°C water bath for 5 minutes to disperse S^0. The sample is periodically agitated during heating. Water is added to bring the final volume to 80 ml, and the sample is agitated until the

contents are evenly dispersed, about 30 seconds. An Alka-Seltzer tablet is added to deaerate flask and capped as described above (Apparatus #2, Figure 2.2). After 5 min. the lid is removed, DTT is added to yield a final concentration of 2mM. An Alka-Seltzer tablet is added, and the lid immediately replaced. A second tablet is added after 5 min. After bubbling subsides, the H_2S concentration is determined by the length of color change along the colorimetric H_2S detection stick.

Linearity, Lower Limit of Detection (LLOD) and Quantification (LLOQ)- The linear range was determined on water samples (5 g) for three commercial Gastec tubes at appropriate concentrations of PEG 400-dissolved S^0-E for each tube: 4LT (0, 0.01, 0.02, 0.03, 0.05 and 0.1 µg g^{-1}) 4LL (0, 0.1, 0.2, 0.5, 1.0 and 2.0 µg g^{-1}) and 4H (0, 2, 4, 6, 12, 24, 48 and 100 µg g^{-1}). Each concentration was run in replicate (n=5). The signal independent noise (σ_i) was determined by Pallesen's method. The LLOQ was defined as $10*\sigma_i$ the LLOD was defined as $3*\sigma_i$.

Evaluation of Potential Interferences- Analyses of grape macerate were run in duplicate using the optimized methodology and 4LT detection tubes. Prior to analysis, grape macerates were spiked with one of two suspected interferences: SO_4^{2-} in the form of $CaSO_4$ (560 mg L^{-1} as SO_4^{2-}) or HSO_3^- in the form of potassium metabisulfite (1000 mg L^{-1} as SO_2).

S^0 recovery from complex matrices: grapes and drywall- Chardonnay grapes were sourced from a local vineyard (Geneva, NY) and homogenized in a Waring blender with an equal weight of water. Niagara grape juice (Welch's, purchased at local supermarket) was used for the juice matrix. Drywall samples were purchased from Home Depot (Pittsburgh, PA), the paper backing removed, and the samples pulverized prior to use. Recovery spikes were analyzed with S^0-E (2, 5 and 10 µg g^{-1} as S^0) in juice and both S^0-E and S^0-MS (2, 5 and 10 µg g^{-1} as S^0) in grape samples. Drywall recovery spikes of S^0-E and S^0-CS were also evaluated. One gram samples were used for juice and grape recovery experiments, and 1

and 2.5 g samples for drywall recovery experiments. Recovery spikes were performed in triplicate with a 4LL detection tube.

Statistics- Minitab and SAS JMP were used for statistical analysis. Normalized coefficients of variance (%CV) were calculated as the standard deviation divided by the mean. Difference of means testing was performed by Tukey HSD.

Results and Discussion

Comparison of Apparatus for H_2S Detection – Initial methods were developed using an apparatus similar to that described by Park (Apparatus #1,): H_2S was sparged from a sample by an external gas source. By using Alka-Seltzer tablets for buffering, de-aerating, and sparging samples, the external gas source could be eliminated (Apparatus #2,Figure 2.2). Furthermore, analysis times for H_2S could be reduced to 15 minutes (data not shown) versus ≥60 min in previous reports [34, 88], likely because of the smaller bubbles and improved mass transfer achieved with the tablets. Linear responses were achieved for H_2S calibration standards in concordance with manufacturers' claims and a previous report [88]. Using reagent grade potassium bicarbonate and citric acid in place of Alka-Seltzer tablets yielded similar results, but was less convenient and also resulted in a more rapid evolution of CO_2, which can dislodge the detection tube or, if large amounts of reagent are used, cause the apparatus to explode. Both manufacturers' detection tubes were found to be effective at measuring H_2S, but the Gastec tubes were utilized for method development due to their wider dynamic range compared to the Sulfur Stick™.

Evaluation of Reducing Agents - The ability of thiols to reduce S^0 to H_2S has been reported in both abiotic and enzymatic systems [28, 89] but this concept has not been utilized previously in a selective method for converting S^0 to S^{2-} for ultimate S^0 quantification. Reducing agents were screened by addition to S^0-E calibration standards under alkaline conditions, and the H_2S evolved quantified in acidic conditions using

the Gastec tubes. DTT was found to efficiently convert S^0-E to H_2S, with a recovery of 109 ± 9.2% relative to that for sulfide calibration standards obtained under optimized assay conditions. The recovery achieved with monothiols (20 mM glutathione or 20 mM cysteine) was 18% and 14%, respectively, of the recovery with DTT under non-optimized conditions (Table 2.2). Recovery with these monothiol reagents did not improve with prolonged reaction time (> 15 min) and improved only slightly with a 10 fold increase in reducing agent concentration. Previous work on the reaction of glutathione with S^0 had observed a similar conversion rate of S^0 to S^{2-} of ~20% [28]. Monothiol reducing agents are reported to yield mixed disulfides when combined with other thiols *in vitro,* resulting in formation of mixed di- or trisulfides (or larger polymers) and non-quantitative recovery of H_2S from S^0 [89]. By comparison, DTT forms a stable cyclic disulfide upon oxidation [90]. The expected reaction between DTT and S_8 is shown in Figure 2.3. TCEP, a common alternative to DTT for reducing disulfide bonds, resulted in poor recovery (9%). Mercaptoethanol was determined to be unacceptable, as it is semi-volatile and interfered with the detection tubes at the high concentrations employed.

Table 2.2. Conversion of S^0 to S^{2-} with Different Reducing Agents

Reducing agent	% conversion	SD
DTT	100	6.4
Glutathione	18.2	1.8
Cysteine	13.6	1.3
TCEP	8.9	1.4
β-mercaptoethanol	N/A [b]	

[a] % conversion calculated as (mol H_2S detected mol^{-1} S^0 added), normalized to DTT recovery (100%). The % conversion for DTT with the optimized methodology was 109%±9. [b] β-mercaptoethanol resulted in interferences on sulfur sticks and conversion could not be determined.

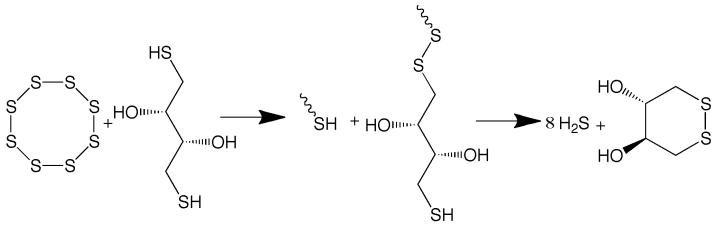

Figure 2.3. Overview of reaction where S_8 is reduced to H_2S by DTT based on the mechanism proposed for reduction of sulfur-sulfur bonds [90, 91].

Optimization of pH - In the initial evaluation of reducing agents, the reduction step was performed under alkaline conditions (pH 11) to increase the concentration of the thiolate forms of DTT (pK_a = 9.2, 10.1). However, the quantification step requires low pH to favor volatilization of H_2S (pK_a =7). Using DTT, we investigated the appropriateness of performing both reduction and quantification steps at a single pH. Near-quantitative recovery was observed when concurrent reduction/quantification was performed at pH 6, and >80% recovery was achieved across the range of pH 5-7 (Figure 2.4). A similar optimum pH range (approximately 5.5-7) has been reported previously for the reaction of glutathione with S^0 [28]. For pH >7, recovery dropped precipitously, likely because HS^- species were favored at these higher pH values, resulting in poor mass transfer to the detection tube. Conveniently, Alka-Seltzer tablets are buffered to pH = 6.05, and thus the target pH can be achieved by adding an Alka-Seltzer tablet to the diluted sample, with the additional benefit of simultaneously deaerating the sample and removing endogenous volatile interfering compounds (e.g., H_2S) prior to addition of the reducing agent.

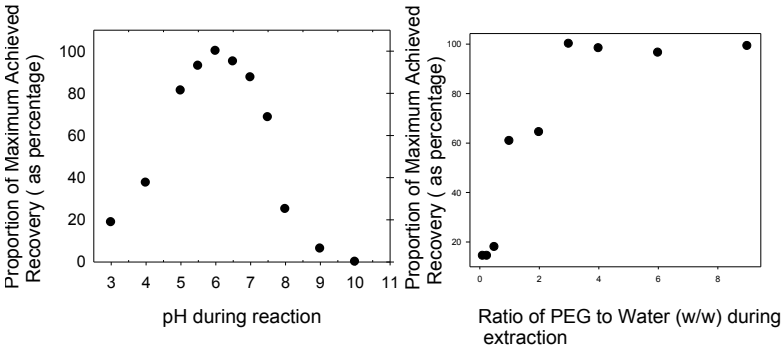

Figure 2.4. (left) Recovery of H_2S after incubation of S^0 with DTT at different pH values. Recovery is reported as a percent of the maximum value achieved. (right) Recovery of H_2S following pre-extraction of S^0-MS in water for 5 minutes, at different dilutions of PEG 400 in water.

Optimizing dispersion of S^0 in PEG 400- In initial trials with recovery spikes, the recovery of S^0-MS was only ~20% of that which could be achieved with S^0-E calibration standards and the theoretical maximum, even when water was used as the matrix (data not shown). Because the major difference in these two experiments was that S^0-E was dispersed in PEG 400 prior to addition of the reducing agent, we adopted an initial step in which the sample is first combined with PEG 400, which has been described elsewhere as an effective co-solvent for dispersing S^0 [86]. While several previous assays have utilized water-immiscible (and hazardous) solvents like CS_2, CCl_4, and toluene for extraction of S^0, quantifying S^0 directly in the sample allows a water-miscible and safer co-solvent (i.e., PEG 400) to be used instead. Using S^0-MS (an S^0 containing fungicide), we obtained maximum recovery with a ratio of 1 part sample per >3 parts PEG 400, and then incubated the mixture at temperatures between 65-100°C for 5 min. prior to the reduction/quantification steps (Figure 2.3). To ensure samples were well within the optimum ranges, a sample:PEG 400 ratio of 1:4 and extraction temp of 80°C were used.

Linearity, LLOQ/LLOD, and Comparison to Other Methods- Linear ranges, LLOQ, LLOD, and other figures of merit for each detection tube are summarized in

Table 2.3. Good linearity ($r^2 > 0.99$, average CV < 10%) was achieved over an order of magnitude for the 4LL (0.12-4.0 µg g^{-1}) and 4H (6-100 µg g^{-1}) detection tubes. The linear range for the 4LT tubes was more limited (0.03-0.10 µg g^{-1}). By selecting an appropriate detection tube, a linear range from 0.03-100 µg g^{-1} in 5 g of buffer could be achieved, i.e., 0.1-500 µg of S^0. Using Pallesen's method, the LLOQ was calculated for 4LT (0.03µg g^{-1}), 4LL (0.12µg g^{-1}) and 4H (6.0 µg g^{-1}) detection tubes for a 5 g sample. Similar LLOQ were achieved for the 4LL tubes with S^0-MS additions to grape samples (data not shown). The LLOQ of 4LL and, especially, the 4LT tubes was limited by background signal, likely due to interferences from endogenous S^0 in the Alka-Seltzer tablets, described in more detail below. Even with this caveat, we can achieve a LLOQ well below 10 µg g^{-1} S^0 with our optimized methodologies using either 4LL or 4LT tube thresholds, the concentration associated with potential H_2S formation in drywall [74] and winegrapes [16]. The detection limits of our method are comparable to or better than other wet chemical and chromatographic methods despite its minimal time and equipment requirements [48, 79, 80, 83](Table 2.1). For example, the lowest LLOQ previously reported was with GC-MS (LLOQ = 0.1 µg g^{-1}) [48], but this approach requires both specialized equipment and organic solvent extraction/pre-concentration prior to analysis. Wet chemical methods generally achieve poorer LLOQ, e.g., oxidation of S^0 to $Fe(SCN)_6^{3-}$ followed by colorimetric detection achieves an LLOQ of 0.8 µg g^{-1}, while also demanding toxic reagents

Table 2.3. Detection Limit and Quantification Ranges for Detection Tubes

Detection tube	Reagent	LLOD ($\mu g\ g^{-1}$)[a]	Linear range ($\mu g\ g^{-1}$)[a]	Linear regression [b]	Correction (mm)[b]	%CV [d]	r^2
Gastec 4LT	$HgCl_2$	0.01	0.03-0.10	0.0127x - 0.276	21.7	9%	0.998
Gastec 4LL	$Pb(CH3COO)_2$	0.036	0.12-4.0	0.234x - 0.435	1.9	7%	0.997
Gastec 4HT	$Pb(CH3COO)_2$	1.81	6-100	9.20x + 2.91	0	7%	0.996

[a] Lower Limit of Detection, determined with 5 g water samples and S^0-E spikes.

[b] Best fit line for linear regression of "mm tube darkened" vs. "ug g^{-1} sulfur"

[c] Correction value accounts for background signal inherent in the test with no S^0 addition, calculated from the x-axis intercept, due to interference from the Alka-Seltzer tablet.

[d] %CV (mean %RSD) calculated for calibration points within quantification range.

and do not selectively reduce S^0 [75, 79]. Our strategy of reducing S^0 to S^{2-} prior to quantification has been previously described, but the reducing agents employed are less desirable. For example, S^0 can be reduced to S^{2-} by Cr^{2+}, which poses safety concerns as well as poor recovery under some conditions; Cu^0 first requires an acetone extraction [44]; and hydrazine hydrate [80] has health and safety concerns related to its use. In addition, Cr^{2+} has been demonstrated to reduce sulfate to H_2S, which would be problematic with both drywall and grape samples [92]. Measurement of S^{2-} by sulfide tube technology was adopted instead of other methods such as sulfide ion-specific electrode [93] and sulfide traps [79] for a variety of reasons, including low cost, ease of use, lower limits of quantification, and better selectivity compared to one or both of these alternatives. In summary, our current approach requires no extraction or pre-concentration steps, minimally toxic reagents, and no specialized equipment while still achieving detection limits comparable to the best existing methods.

Interferences- SO2 can reportedly interfere with the performance of sulfide detection tubes; however, at the optimized pH range (pH = 5-7), SO2 exists primarily as non-volatile HSO3-, and does not interfere with analyses. With our optimized methodology, we observed no interference on the 4LT tubes with

spikes of SO42- in the form of gypsum (560 mg L-1 as SO42-) and HSO3- in the form of potassium metabisulfite (1000 mg L-1 as SO2). The HgCl2 based tubes (4LT) are reported to react with methyl mercaptan [88], and the presence of endogenous mercaptans or H2S could yield incorrectly high measurements. Additionally, O2 in the sample or buffer could oxidize H2S and reduce recovery. These problems are avoided by the initial addition of an Alka-Seltzer tablet to simultaneously degas and buffer the sample prior to addition of the reducing agent. However, this step could also convert disulfides to mercaptans, again resulting in interferences for the HgCl2 tubes. Thus, as a general caveat, we would not recommend using HgCl2 based sulfide detection tubes in cases where interferences from mercaptans are possible. Finally, we observed a small signal in blanks, equivalent to 0.05 µg g-1 S^0

Table 2.3). Substitution of reagent grade potassium bicarbonate and citric acid in place of Alka-Seltzer tablets yielded no detectable interference, suggesting that the tablets likely contain a small amount of S^0 impurity.

S^0 Recovery in Real Matrices - Recovery spikes of S^0 in grape and juice samples (2, 5, 10 μg g^{-1} added to 1 g samples, n = 5) were evaluated (Table 4). Recoveries ranged from 90-95% for S^0-E and from 82-88% for S^0-MS. The recovery spikes of S^0 used were at representative concentrations for residues reported to cause production of off-aromas during fermentations [16, 26]. We observed similarly good recoveries (>90%) for spikes of 1, 5 and 10 μg g^{-1} S^0-E and 4 and 10 μg g^{-1} of S^0-CS into 1 g of drywall matrix, where 10 μg g^{-1} has been suggested as a limit for S^0 in drywall [74]. Recovery of S^0-E spikes to 2.5 g drywall samples (0.8 and 2.4 μg g^{-1}) was non-quantitative, ~70%, although good reproducibility was achieved. While it is unclear at this time why a high ratio of drywall to solvent results in reduced recovery, we would advise evaluating recovery prior to extending our assay to new matrices.

Table 2.4. Recovery of S^0 Spikes from Complex Matrices

Matrix	Sulfur form	Sulfur spike ($\mu g\ g^{-1}$)	Recovery (%, mean)	CV (%)
Juice (1 g)	S^0-E	2, 5, 10	93.4	7.4
Grapes (1 g)	S^0-E	2, 5, 10	92.7	9.8
Grapes (1 g)	S^0-MS	2, 5, 10	84.7	10.2
Drywall (2.5g)	S^0-E	0.8, 2.4	74.9	8.1
Drywall (1 g)	S^0-E	1, 5, 10	98.5	9.3
Drywall (1 g)	S^0-CS	4, 10	106.6	6.9

Conclusion

The S^0 quantification assay reported here represents an inexpensive and convenient alternative to existing methodologies. The equipment cost is <$50 and the cost of consumables is ~$10/run, with potentially lower costs achievable by recycling detection tubes. Individual analyses require <15 minutes each, can be performed with minimal laboratory facilities and can be learned by unskilled practitioners with minimal training. The waste generated is mostly benign, although the microgram quantities of Hg or Pb in the sulfide detection tubes may require special disposal in some regions. Limits of quantification are comparable to the best reported from chromatographic and colorimetric methods, despite requiring no pre-concentration, extraction, or specialized equipment and can be further improved by using high purity reagents in place of the more convenient Alka-Seltzer tablets. Acceptable recovery could be achieved in diverse matrices, and the method appears to be sufficiently robust and accurate for general use in quantification of S^0 in environmental samples. However, although good recovery could be achieved for low drywall:solvent ratios, weaker recovery (~70%) was achieved for a high drywall:solvent ratio,

indicating that the method will likely need validation when extended to novel matrices. Finally, we see potential for adopting the methodology into more convenient colorimetric tests for semi-quantitative or qualitative analyses of S^0 in complex samples, e.g., with colorimetric test-strips.

References

1. Rankine, B.C. Nature, origin and prevention of hydrogen sulphide aroma in wines. *J. Sci. Food Agric.* **1963,** *14,* 79-91.

2. Acree, T.E.; Sonoff, E.P.; Splittstoesser, D.F. Effect of yeast strain and type of sulfur compound on hydrogen sulfide production. *Am. J. Enol. Vitic.* **1972,** *23,* 6.

3. U.S. Consumer Product Safety Commission and U.S. Department of Housing and Urban Development Interim Guidance – Identification of Homes with Corrosion from Problem Drywall – Revision 1. **2010,**

4. Troelsen, H.; Jørgensen, B.B. Seasonal dynamics of elemental sulfur in two coastal sediments. *Estuar. Coast. Shelf Sci.* **1982,** *15,* 255-266.

5. Hurse, T.J.; Abeydeera, W.P.P. Quantification of sulfur and sulfur-containing compounds in wastewaters by means of a combination of liquid chromatographic methods. *Journal of Chromatography A* **2002,** *942,* 201-210.

6. Pauls, R.E. Determination of Elemental Sulfur in Gasoline by Gas Chromatography with On-Column Injection and Flame Ionization Detection Following Derivatization with Triphenylphosphine. *J. Chromatogr. Sci.* **2010,** *48,* 283-288.

7. Crawford, C.L.; Boudries, H.; Reda, R.J.; Roscioli, K.M.; Kaplan, K.A.; Siems, W.F.; Hill Jr, H.H. Analysis of Black Powder by Ion Mobility− Time-of-Flight Mass Spectrometry. *Anal. Chem.* **2009,** *82,* 387-393.

8. Chen, Y.W.; Joly, H.A.; Belzile, N. Determination of elemental sulfur in environmental samples by gas chromatography-mass spectrometry. *Chem. Geol.* **1997,** *137,* 195-200.

9. Kamyshny Jr, A.; Borkenstein, C.G.; Ferdelman, T.G. Protocol for Quantitative Detection of Elemental Sulfur and Polysulfide Zero-Valent Sulfur Distribution in Natural Aquatic Samples. *Geostandards and Geoanalytical Research* **2009,** *33,* 415-435.

10. Luther III, G.W.; Giblin, A.E.; Varsolona, R. Polarographic analysis of sulfur species in marine porewaters. *Limnol. Oceanogr.* **1985,** 727-736.

11. Bartlett, J.K.; Skoog, D.A. Colorimetric determination of elemental sulfur in hydrocarbons. *Anal. Chem.* **1954,** *26,* 1008-1011.

12. Fossing, H.; Jørgensen, B.B. Measurement of bacterial sulfate reduction in sediments: evaluation of a single-step chromium reduction method. *Biogeochemistry* **1989,** *8,* 205-222.

13. Hardman, A.F.; Barbehenn, H.E. Determination of free sulfur in rubber. *Industrial & Engineering Chemistry Analytical Edition* **1935,** *7,* 103-104.

14. Berner, R.A. Distribution and diagenesis of sulfur in some sediments from the Gulf of California. *Mar. Geol.* **1964,** *1,* 117-140.

15. Cline, J.D. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **1969,** *14,* 454-458.

16. Henshaw, P.F.; Bewtra, J.K.; Biswas, N. Extraction of elemental sulfur from an aqueous suspension for analysis by high-performance liquid chromatography. *Anal. Chem.* **1997,** *69,* 3119-3123.

17. Fabbri, D.; Locatelli, C.; Tarabusi, S. A new procedure, based on combustion to sulphate and ion chromatography for the analysis of elemental sulphur in sediments. *Chromatographia* **2000,** *53,* 119-121.

18. Wang, L.H.; Chin, C.Y. Determination of sulfur in cosmetic products using ion-selective electrode. *Journal of Analytical Chemistry* **2007,** *62,* 688-690.

19. Howarth, R.W.; Jørgensen, B.B. Formation of 35S-labelled elemental sulfur and pyrite in coastal marine sediments (Limfjorden and Kysing Fjord, Denmark) during short-term 35SO42-reduction measurements. *Geochim. Cosmochim. Acta* **1984,** *48,* 1807-1818.

20. Nriagu, J.O.; Soon, Y.K. Distribution and isotopic composition of sulfur in lake sediments of northern Ontario. *Geochim. Cosmochim. Acta* **1985,** *49,* 823-834.

21. Azarova, I.N.; Gorshkov, A.G.; Grachev, M.A.; Korzhova, E.N.; Smagunova, A.N. Determination of elemental sulfur in bottom sediments using high-performance liquid chromatography. *Journal of Analytical Chemistry* **2001,** *56,* 929-933.

22. Henshaw, P.F.; Bewtra, J.K.; Biswas, N. Extraction of elemental sulfur from an aqueous suspension for analysis by high-performance liquid chromatography. *Anal. Chem.* **1997,** *69,* 3119-3123.

23. Luther III, G.W.; Church, T.M. Seasonal cycling of sulfur and iron in porewaters of a Delaware salt marsh. *Mar. Chem.* **1988,** *23,* 295-309.

24. Brenner, M.W.; Owades, J.L. Stable Colloidal Sulfur Solutions. *Science* **1954,** *119,* 911.

25. Park, S.K. Development of a method to measure hydrogen sulfide in wine fermentation. *Journal of microbiology and biotechnology* **2008,** *18,* 1550-1554.

26. Hach Company Hydrogen Sulfide Test Kit, Model HS-C, 0-5 mg/L, 18 tests. **2010,** *2010,* 1.

27. Ugliano, M.; Henschke, P.A. Comparison of three methods for accurate quantification of hydrogen sulfide during fermentation. *Anal. Chim. Acta* **2009,** *660,* 87-91.

28. Searcy, D.G.; Lee, S.H. Sulfur reduction by human erythrocytes. *Journal of Experimental Zoology Part A: Comparative Experimental Biology* **1998,** *282,* 310-322.

29. Sluiter, E. The production of hydrogen sulphide by animal tissues. *Biochem. J.* **1930,** *24,* 549.

30. Cleland, W.W. Dithiothreitol, a New Protective Reagent for SH Groups*. *Biochemistry (N. Y.)* **1964,** *3,* 480-482.

31. Singh, R.; Lamoureux, G.V.; Lees, W.J.; Whitesides, G.M. Reagents for rapid reduction of disulfide bonds. *Methods Enzymol.* **1995,** *251,* 167-173.

32. Mylon, S.E.; Hu, H.; Benoit, G. Unsuitability of Cr (II) reduction for the measurement of sulfides in oxic water samples. *Anal. Chem.* **2002,** *74,* 661-663.

33. Searcy, D.G.; Peterson, M.A. Hydrogen sulfide consumption measured at low steady state concentrations using a sulfidostat. *Anal. Biochem.* **2004,** *324,* 269-275.

34. Schutz, M.; Kunkee, R.E. Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast. *Am. J. Enol. Vitic.* **1977,** *28,* 137.

Chapter 2 Reprinted from Analytica Chimica Acta, Vol. 703, Misha T. Kwasniewski, Rachel B. Allison,Wayne F. Wilcox,Gavin L. Sacks, Convenient, inexpensive quantification of elemental sulfur by simultaneous in situ reduction and colorimetric detection , Pages No. 52-57, Copyright (2011), with permission from Elsevier

Chapter 3

PERSISTENCE OF ELEMENTAL SULFUR SPRAY RESIDUE ON GRAPES DURING RIPENING AND VINIFICATION

Abstract

Increased hydrogen sulfide (H_2S) production during fermentation can occur when elemental sulfur (S^0) applied in the vineyard persists into the fermentation. Utilizing a rapid, inexpensive method for quantifying S^0, residue was monitored in the vineyard and during vinification over 3 years of field studies. The S^0 formulation, application rate, and timing of the last application before harvest all affected S^0 residue concentrations on the fruit at harvest. In all years, ceasing application at least 35 days prior to harvest resulted in levels <10μg/g. Applications closer to harvest generally resulted in higher residue levels, which transferred proportionally to the musts after pressing. However, very little S^0 was detectable in white wine fermentations when the grapes were pressed and the must clarified through settling prior to fermentation, regardless of the initial residue levels on harvested fruit.

Interpretive abstract

Elemental sulfur (S^0) is a commonly used pesticide that is used primarily for the control of powdery mildew. While there are many benefits to S^0 compared with alternatives, including cost, and the lack of resistance development risk, S^0 is still used sparingly by many growers late in the season due to the risk of off aromas in the finished wine. Concentrations of S^0 above 10µg/g have consistently been tied to increased H_2S production during fermentation. Despite the economic importance of the relationship between S^0 and faulted wine limited research has been conducted to understand when S^0 applications should cease to avoid faulted wine. This has in part due to the lack of a simple method for S^0 quantification. Three years of field trials in the Finger Lakes Region of NY State were conducted, during which S^0 was applied late into the season and quantified using an assay appropriate for use industry or laboratory use. Over the three years treatments included application of different formulations (≈1 or ≈10µ particle size), different applications rates, as well as varying the timing between last application S^0. In all cases ending S^0 applications 35 days or more prior to harvest resulted in S^0 levels below 10µg/g with some treatments obtaining levels below 10µg/g as close as 22 days before harvest. S^0 levels in treatments ending within 35 days prior to harvest ranged between approximately 6-52 µg/g depending on formulation, timing and application rate. While formulation and application rate effected S^0 residue concentration and persistence for some treatments, timing between final treatment and harvest consistently had the largest effect on final concentrations. Applications closer to harvest resulted in higher S^0 concentrations on the fruit. The concentration of S^0 that persisted into the fermentation was effected by vinification decisions. Residue concentrations were reduced after pressing and settling the juice.

Key Words: Pesticide, Fungicide, Reduced, Aroma, Quantification, Powdery Mildew

Introduction

Elemental sulfur (S^0) is an effective and inexpensive control for the most common disease of grapes worldwide, powdery mildew (PM), caused by the fungus *Erysiphe necator* (syn. *Uncinula necator*) [1]. Various commercial formulations of S^0 are used extensively for this purpose, not only for its cost and efficacy attributes, but also because the pathogen is unlikely to develop resistance to this fungicide and because it is permitted in various "organic" and "biological" production systems, where it is perhaps the most efficacious treatment available [2]. However, S^0 residues still present at harvest can be reduced to hydrogen sulfide (H_2S) during fermentation, and its use in the vineyard has been tied to reduced sulfur characters in the finished wines made from treated grapes [3-6]. Unfortunately, there are few data available concerning the persistence of S^0 in the vineyard and during vinification that allow growers and winemakers to objectively assess this risk of late season sulfur applications, sometimes resulting in arbitrary commercial restrictions and conflicting recommendations relative to the minimum preharvest withholding period for the fungicide. A poor understanding of this relationship increases the likelihood of economic losses resulting from both (i) an unnecessary overreliance on more expensive alternatives to S^0, which oftentimes also increases the probability of compromised disease control following the eventual development of pathogen resistance to these substituted materials; and (ii) the production of faulted wine as a result of S^0 application too close to harvest.

While a number of studies into the relationship between S^0 and H_2S have included either a vineyard or vinification component, only a very limited number have attempted to quantify S^0 residue following treatment in the field and relate these values to H_2S production during fermentation. Furthermore, there are conflicting data among the limited studies attempting to quantify S^0 persistence in the field. For example, Thomas et al. (1993) working in California found that applications of 10 to 17 kg/ha of S^0 applied as dust resulted in residues <14 µg/g of S^0 on fruit 1 day after application, that these had declined to <4 µg/g within 2 additional weeks, and that final concentrations at harvest (6 weeks after the last application) were 1 to 3.4 µg/g. In contrast, Wenzel et al. (1980) working in Germany found

residue levels as high as 8 μg/g at harvest when applications of a sprayable formulation of S^0 ceased 7 weeks prior to harvest [7], although application rates were not disclosed. In this and a previous study (Wenzel and Dittrich, 1978), the same group also demonstrated that clarification of white wine must can greatly reduce S^0 levels therein, leading to a reduction in H_2S production during fermentation [8].

A major impediment to studies requiring quantification of S^0 residues has been the lack of an affordable technology to do so in complex matrices such as grape juice and must, as economical techniques typically measure total sulfur, including that in endogenous sulfates, amino acids, etc. in addition to S^0. Thomas et al. (1993) circumvented this limitation by washing sulfur dust residues from the surface of intact clusters and measuring total S in the rinsate [9]. However, we were unable to apply this technique successfully in our own initial field studies, as the sprayable formulations of S^0 utilized in many regions, including humid climates such as New York, still left visible residues on the fruit after repeat washings, and measured S levels in the rinsate were unexpectedly low.

Thus, before proceeding any further, I developed a rapid, inexpensive technique for measuring S^0 in complex matrices, based upon its quantitative reduction to H_2S *in situ* and simultaneous colorimetric quantification using commercially available detection tubes [10]. The chapter reports the subsequent use of this technique to study the effect of fungicide formulation, rate, and application timing on the persistence of S^0 residues on grape clusters in the field and their transfer to the must after harvest and crushing. Additionally, we report upon the influence of vinification factors such as whole-cluster pressing, length of skin contact, and must clarification on S^0 persistence. Brief portions of this work have been published previously [11-14] (cite any relevant abstracts from AJEV or other accessible journals).

Material and Methods

Elemental Sulfur Quantification. Details of the quantification procedure are provided by Kwasniewski et al. (2011) (Chapter 2 of this thesis). Briefly, for intact grape samples from the field, a whole cluster (either freshly harvested or frozen for storage) was first blended with an equal weight of water using an

immersion blender; juice and must samples obtained after pressing were not blended or diluted. Following dispersion of S^0 in heated PEG 400, the sample was then diluted with water and subsequently de-aerated and adjusted to pH 6 through the addition of a pharmaceutical tablet designed to evolve CO_2 in aqueous media and buffer acidic solutions (Alka-Seltzer, Bayer Healthcare, Morristown, NJ). Following de-aeration, dithiothreitol (Fisher Scientific, Pittsburgh, PA) was added to promote reduction of S^0 to H_2S. Resulting H_2S was sparged through a detection tube with sequential addition of more such tablets, and the quantity of elemental sulfur contained in the sample was determined by relating the distance of color change in the H_2S detection tube to a calibration curve.

Elemental sulfur persistence following field applications. Three years of field trials were conducted in test vineyards at the New York State Agriculture Experiment Station in Geneva, NY (lat.: 42°52'43"; long.: -77°0'56"), to determine the effect of time, product formulation, and application rate on S^0 persistence. In 2009 and 2010, these trials were conducted on vines of *Vitis vinifera* cv. Chardonnay, and in 2011 on *V. vinifera* cv. Riesling vines. All vines were planted in 2004 on 3309C rootstock, and were trained to a vertical shoot-positioned system with 3-m row spacing and 2-m vine spacing. Vines were sprayed and fertilized as per normal commercial practices for the region, except that no S^0 sprays were applied other than those in the variable treatment regimens. S^0 treatments were applied to test vines using an over-the-row, hooded boom sprayer, operating at a pressure of 2070 kPa and delivering a water volume of 935 L/ha.

In 2009, a single application of a micronized formulation of S^0 (Microthiol Disperss 80DF, Cerexagri Inc,. King of Prussia, PA), was made either 12, 40 or 68 days pre-harvest, at a rate of either 2.69 or 5.38 kg/ha of S^0. Each of the 7 treatments, including a control, were applied in a randomized complete block design to six, four-vine panels, including a control in which no S^0 was applied. Micronized dry flowable S^0 formulations like Microthiol Disperss have a particle size of approximately 4µm diameter [15].

In 2010, all treatments were initiated on August 12 (veraison, approximately), with additional sprays applied at approximately 2-week intervals and continuing until either 50, 35, 22, or 8 days before harvest, depending on the treatment. Vines in the latter three timing regimens received applications of either (i) Microthiol Disperss, at a 2.69 or 5.38 kg/ha application rate of S^0; or (ii) a wettable sulfur formulation (Kumulus DF Cary, NC), providing 5.38kg/h of S^0; vines in the 50-day pre-harvest treatment received only a single application of Microthiol at the 2.69kg/ha application rate. According to the manufacturer, Kumulus DF has a particle size approximately of 0.1 to 8 µm, with a mean diameter of approximately 4 µm diameter. Individual plots consisted of two consecutive four-vine panels for each of the 11 treatments (including control), arranged in a randomized complete block design with three replications. Five clusters were randomly sampled from each panel at 32, 30, 28, 24, 20, 16, 7, 2 and 0 days before harvest. The five-cluster sample was blended and the concentration of S^0 in each sample quantified using the above protocol resulting in n=6 for each treatment at each time point. Values were first compared within a given sampling date using 2-Way ANOVA, followed by parametric testing within a sampling period using Tukey HSD.

The 2011 treatments began on July 13, 2011 with all treatments initially receiving either 4.48kg/ha of Microthiol Disperss or Kumulus DF. Individual plots consisted of two consecutive four-vine panels, with the 10 treatments (including control) arranged in a randomized complete block design, with three replications,. This resulted in 6 replicate panels for each treatment and a total of 60 four vine panels. Panels received either 4.48kg/ha of Microthiol Disperss or Kumulus DF at approximately 2-week intervals until 12, 25, 38 or 54 days before harvest. An additional treatment was included that received 3 treatments of Microthiol at 4.48kg/ha and its final two treatments, at 2.24kg/ha. Five clusters were randomly sampled from each panel at 62, 53, 47, 40, 31, 24, 17, 9 and 0 days before harvest. The five-cluster sample was blended and the concentration of S^0 in each sample quantified using the above protocol resulting in a n=6 for each treatment at each time point. Values were compared within a given sampling date first using 2-Way ANOVA, followed by parametric testing within a sampling period using

with Tukey HSD.

White wine vinification procedure. All wines were vinified in triplicate using the following procedures, unless otherwise noted. Grapes from a given treatment were hand harvested, crushed and de-stemmed, then pressed in a hydraulic basket press. The collected juice was treated with 50 μg/ml SO_2 and allowed to settle for 24 hours. Following settling, juice was inoculated with *Saccharomyces cerevisiae* strain R-HST yeast (Lallemand, Petaluma, CA) previously rehydrated in 10 μg/ml GoFerm (Lallemand) according to manufacturer's instructions. Nutrient analysis was conducted and soluble solid content was determined by refractometer. Ammonia and alpha-amino acid levels were quantified enzymatically using Unitab reagents and a ChemWell multiscanner prior to inoculation (Unitech Scientific, California U.S.). If necessary, nutrients were added at recommended levels in the form of Fermaid K (Lallemand, Petaluma, CA), to a maxiumum concentration of 25 μg/ml of the latter; $(NH_4)_2HPO_4$ was added if additional nitrogen was required to meet recomendation. Further details on nutrient amendments are outlined below for specific years. Wines were fermented at 10°C to dryness as determined by Clinitest (Bayer, West Haven, CT), cold stabilized at -4°C, and bottled under Stelvin closures (Waterloo Container, Waterloo, NY). Following primary fermentation, wine transfers (i.e., racking and bottling) were made under N_2 gas.

H_2S produced during fermentation was quantified in 2010 and 2011, using a H_2S detection tube to quantify H_2S in escaping [16, 17]. After fermentation in these years, immediately before bottling, H_2S was quantified in duplicate 80-ml samples of all wines produced [10, 18].

In 2009, the vinification procedures described above were amended to provide for must amelioration that was necessary due to berry desiccation from powdery mildew infection. Water was added at a rate of 20 ml/l of must to reduce the soluble solids and titratable acidity from 30.4°(±0.5) Brix and 14.8(±0.3)g/l, respectively, to 24.6°(±0.5)Brix and 11.4(±0.2)g/l, respectively. Following amelioration, nitrogen levels were tested and adjusted to 300 μg/ml.

In 2010, clusters were sorted prior to crushing/destemming due to late-season rot problems, and fruit deemed to be commercially unacceptable was removed. However, in order to obtain harvest-day fruit samples, representative clusters before and after sorting were taken. Soluble solids and titratable acidity of juice were between 20.8°Brix (±0.4) and 8.4 g/l (±0.3) respectively, with pH values of 3.35 (±0.1). Due to poor yield resulting from a combination of late spring frost events and fruit removed during sorting, sufficient fruit did not exist to vinify all treatments. Thus, triplicate 1-l fermentations were made with fruit from all timings of the 5.38kg/ha Microthiol treatments, as well as from the other treatments that ceased 8 days prior to harvest.

No amendments to the basic vinification procedure were necessary in 2011. Each treatment produced triplicate 20-l fermentations, which were fermented to dryness while monitoring H_2S production daily using detection tubes as described above. Samples were taken to measure S^0 residues from the intact fruit prior to processing as well as in the juice prior to settling. To standardize clarity levels of the juice samples used for testing, must turbidity was employed as a metric for effective clarification in 2011. Juice was clarified for 24 hours, and S^0 residue levels and turbidity were determined from samples taken with a wine thief from 30cm below the surface at different time points during the clarification process. In 2011, all clarified must obtained a level of turbidity below 20ntu as measured using a Hach 2100Q Turbidimeter (Hach Company, Loveland, Colarado), and this level was not exceeded following racking. In earlier years the determination of final clarity prior to fermentation was made visually.

Skin contact effect on S^0 persistence and H_2S production. In 2010, a trial was conducted to investigate the persistence and fate of S^0 from the vineyard to the must and on through to finished wines made with variable amounts of skin contact. Fruit was sourced from a commercial vineyard of cv. Cabernet Franc located near Geneva, NY (lat.: 42°50’40”; long.: -77°0’13”), which was established in 2005 on 3309C rootstock with 3-m row spacing and 2-m vine spacing. On 22 September, test vines received a single application of Microthiol Disperss, providing 2.69kg/ha of S^0, using the spray equipment and technique described above.

This trial consisted of 5 treatments vinified in triplicate, whole-cluster pressed; crushed/destemmed and pressed; crushed/destemmed and pressed following 24hours skin contact; crushed/destemmed and pressed following a 1-week on the skins; or crushed-destemmed and pressed following a 2-week maceration on the skins. The whole-cluster treatment consisted of approximately 20% of the fruit from each harvest bin which was removed, pooled, and vinified in triplicate following whole-cluster pressing. The remaining fruit was homogenized, crushed and destemmed, and the macerate was either pressed immediately or allowed to remain in contact with the skins at 4°C for 24 hours before pressing. After pressing, fruit were fermented on the skins for either 7 or 14 days, with the cap reintegrated daily. This fruit was divided into triplicate, pressed into 20-l carboys, then settled and vinified using the above white winemaking procedure. Following crushing/destemming, the collected matter was mixed between a series of 60-l stainless steel containers and divided into 12 aliquots, with three replicate samples assigned to each of four additional treatments. One such treatment was pressed immediately into 20-l carboys, another stored at 4°C for 24 hours prior to pressing in 20-l carboys; after pressing, both were vinified following the white winemaking procedure described above. The grape macerate for the extended skin contact treatments were placed in 25-l plastic pails with air tight lids, then inoculated and their nitrogen levels adjusted as other treatments (Midwest Supplies, Minneapolis, MN). During the 7-day or 14-day maceration period the buckets remained closed but the skins were integrated by swirling the contents of each bucket for about 1 minute daily. After the given period of maceration the red wines were hand pressed through cheese cloth and the juice transferred into a glass carboy.

During fermentation H_2S was monitored by an attached H_2S detection tube. S^0 residue levels were quantified in the juice before and after settling as well as in wine post fermentation and in the lees. Differences between treatments for H_2S released and S^0 residue were parametrically tested by Tukey HSD following 1-way ANOVA. The relationship between S^0 in the different juice and wine samples to H_2S was investigated using linear regression.

Statistics. SAS .JMP version 9.0.2 was used for all statistical analysis (SAS Cary, NC). Parametric mean testing was done using Tukey HSD, following confirmation by ANOVA of differences.

Results

Residue levels on grapes at harvest. In 2009, application of S^0 12 days prior to harvest (preH), resulted in S^0 residue on the fruit >10x that observed on other treatments (Figure 3.1). Applications that ceased 40 days preH produced residues (near 1 μg/g) that were higher than the control treatment (no measurable residues), but at a level shown to have minimal effects on wine quality (citations). And whereas some samples for the 68 days preH had detectable levels of S^0 residue, the mean value for this treatment could not be differentiated statistically from the control. Two-way ANOVA analysis indicated that the timing of the S^0 application was a contributor to the variance (P <0.0001) whereas the application rate was not. Only fruit treated 12 days before harvest resulted in residue levels above the 10μg/g putative threshold that has been shown to consistently increase H_2S production during fermentation.

In 2010, the variables investigated, S^0 treatment (formulation and rate) and timing of the last application before harvest both impacted final residue levels (Figure 3.1) (P <0.001). All treatments applied 8 days preH resulted in concentrations exceeding 10μg/g, although residues following applications of S^0 at 2.69 kg/ha in a wettable formulation were only about one third the level of those following applications at 5.38 kg/ha in a micronized form; residues following applications at this higher rate in a wettable form were intermediate between those of the two other treatments and the three means were significantly (P <0.05) different from one another (Figure 3.1). When sprays ceased 22 days preH, residues resulting from applications of wettable sulfur at the lower rate averaged 6.4μg/g (±2.6) μg/g, whereas applications of either formulation at the higher rate resulted in significantly (P <0.05) higher levels, well in excess of 10μg/g (Figure 3.1). All three treatments ceasing 35 days preH produced statistically comparable residue levels well below the putative threshold (0.6 to 4.6μg/g), and residues

were negligible when the final spray of S^0 at 2.69 kg/ha in the micronized formulation was made 50 days preH (Figure 3.1).

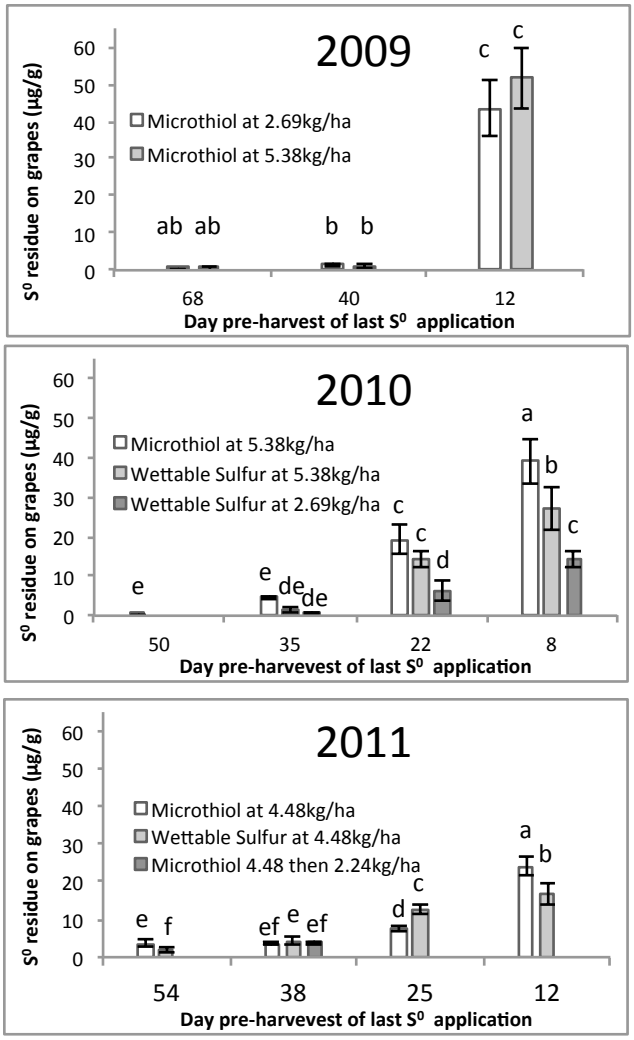

Figure 3.1. S^0residues on Chardonnay (2009, 2010) and Riesling (2011) clusters at harvest. Data are grouped by days before harvest of the final S^0 application, with each bar representing the mean value for a five-cluster sample taken from each of six replicate treatment plots. Means not labeled with a common letter are significantly different (Tukey HSD, P <0.05). No residue was detected on any samples obtained from a control treatment in which S^0 was not applied (data not shown).

In 2011, both the duration of the preharvest interval and the formulation of S^0 affected residue levels on grapes at harvest. For each of the two formulations that provided a constant S^0 rate of 4.48 kg/ha, residues were inversely proportional to the duration of the preharvest interval (PHI), with the

exception that there was no significant ($P< 0.05$) difference between the 38- and 54-day preH cessations for the Microthiol sprays (Figure 3.1). Residues were near or well above 10 µg/g when either formulation was applied until either 25 or 12 days preH; those from Microthiol were significantly ($P< 0.05$) greater than those from the wettable formulation given the shorter PHI, whereas the converse was true for the longer. Residues ranged from 1.9 to 3.7 µg/g given either a 54- or 38-day PHI, regardless of treatment (Figure 3.1).

Persistence and accumulation in the vineyard. Multiple panels sampled on the earliest dates in 2010 had received identical sulfur applications, as these treatments varied only by time between the final application and harvest. Therefore, only those treatments that varied by either formulation or application rate could be differentiated at the early sampling dates, before spray timing also became a factor. For example, across all three PHI treatments that had received identical sulfur applications prior to that sampling date, residue levels 32 days before harvest (i.e., 3 days after the most recent application) averaged 27.1 µg/g for Microthiol at 5.38 kg/ha, 34.4 µg/g for Kumulus at 5.38 kg/ha, and 20.1 µg/g for Kumulus at 2.69 kg/ha; at 30 days before harvest, these levels had decreased to 21.0, 17.1, and 10.3 µg/g, respectively; at 28 days they were 27.6, 9.6, and 8.1 µg/g, respectively; and at 24 days, they were 13.7, 9.6, and 6.9 µg/g, respectively.(Figure 3.2). These effects were even more pronounced immediately following an application and appeared to be cumulative over time. For example, across the two PHI timings that received an application 22 days before harvest, residues averaged 49.6 µg/g for Microthiol at 5.38 kg/ha, 55.9 µg/g for Kumulus at 5.38 kg/ha, and 27.7 µg/g for Kumulus at 2.69 kg/ha; 1 day following the final 8-day PHI treatment, these values were 66.7, 85.9, and 30.0 µg/g, respectively (Figure 3.2). In 2011, a season with significant climatic differences versus the preceding one and in which application rate was not a factor, differences between the two sulfur formulations were inconsistent. As in 2010, residue levels often spiked immediately after treatment, declining by about one half after approximately one week (Figure 3.3). Samples from control panels in which no S^0 was applied were also quantified at all time points in both years; however, specific data are not presented, as residue levels were

always below the limit of detection (0.01 µg/g) for the technique used. Additional detailed data are provided in supplemental-information Table 3.3 and Table 3.4.

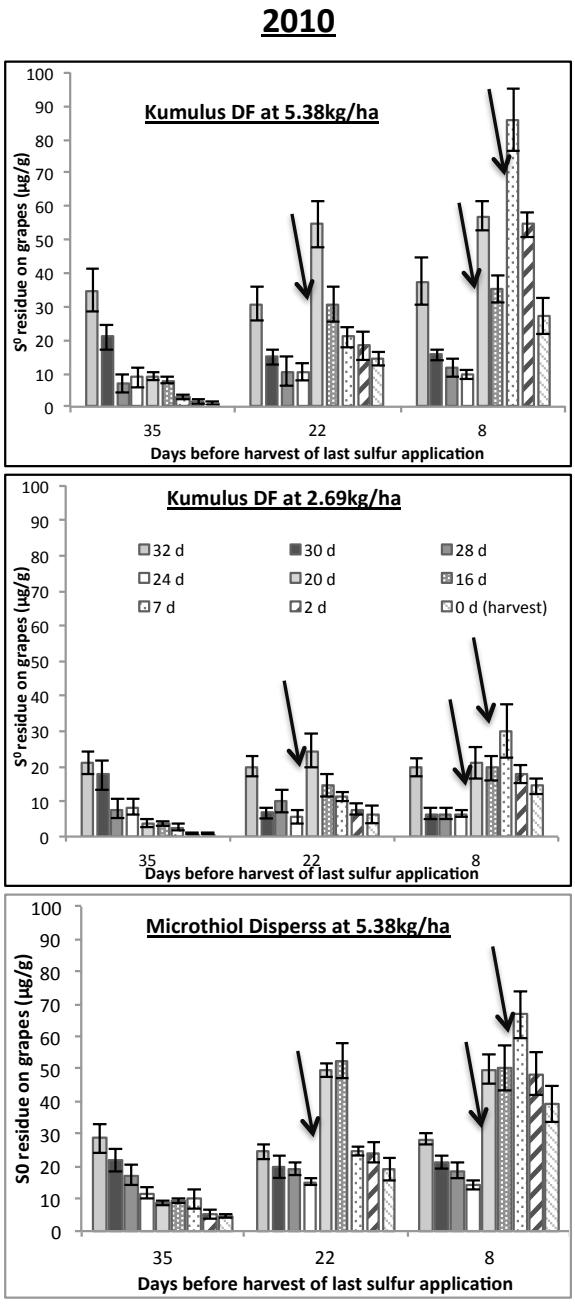

Figure 3.2. Elemental sulfur (S^0) residue on Chardonnay grape clusters sampled throughout the 2010 season. Sequential sprays of commercial sulfur formulations were applied starting 50 days before harvest and continuing at approximately 2-week intervals, ceasing a variable number of days before harvest as denoted on the x-axis. S^0 residue data are grouped by sulfur treatment, with each bar representing the mean value for a five-cluster sample taken from each of six replicate treatment plots. The legend denotes the number of days before harvest that the sample was obtained, and arrows signify when a S^0 application was made during the sampling period. No residue was detected on samples from a control treatment in which S^0 was not applied (data not shown). Detailed data for each sample measurement is contained in the supplemental-information Table 3.2.

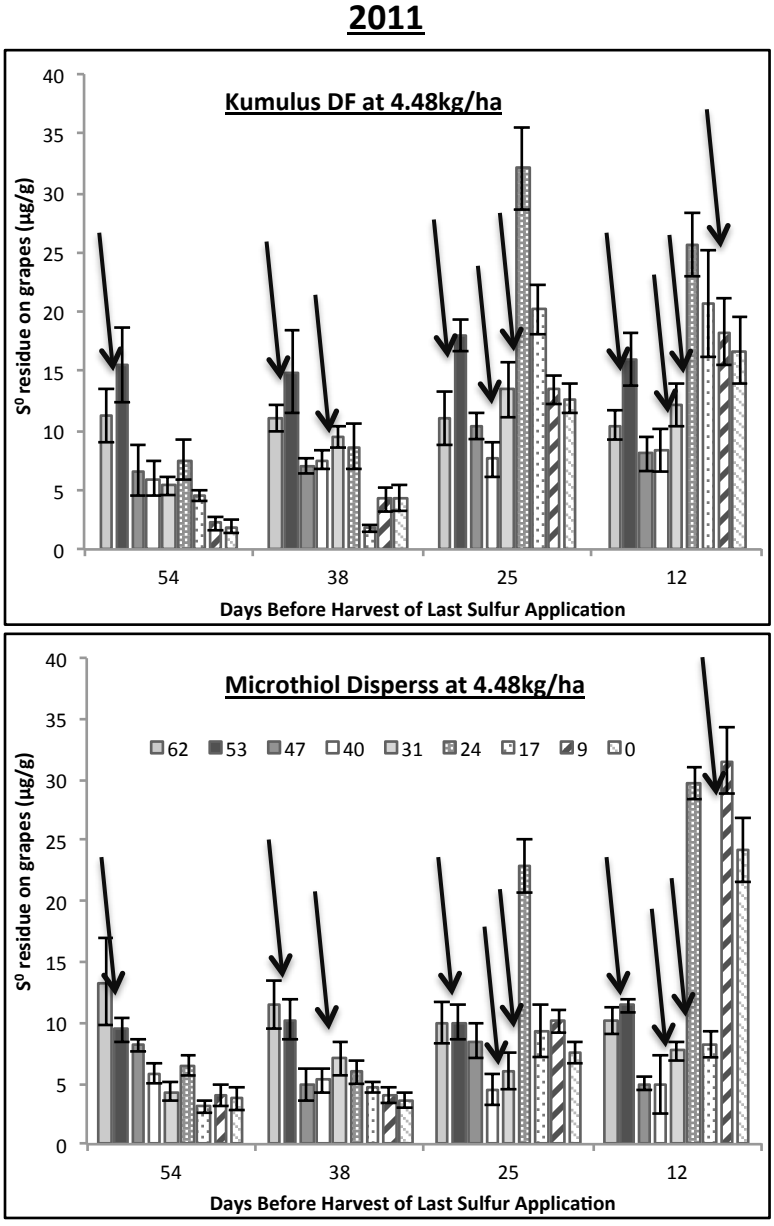

Figure 3.3. Elemental sulfur (S^0) residue on Riesling grape clusters sampled throughout the 2011 season. Sequential sprays of commercial sulfur formulations were applied starting 81 days before harvest and continuing at approximately 2-week intervals, ceasing a variable number of days before harvest as denoted on the x-axis. S^0 residue data are grouped by sulfur treatment, with each bar representing the mean value for a five-cluster sample taken from each of six replicate treatment plots. The legend denotes the number of days before harvest that the sample was obtained, and arrows signify when a S^0 application was made during the sampling period. No residue was detected on samples from a control treatment in which S^0 was not applied (data not shown). Detailed data for each sample measurement is contained in the supplemental-information Table 3.3.

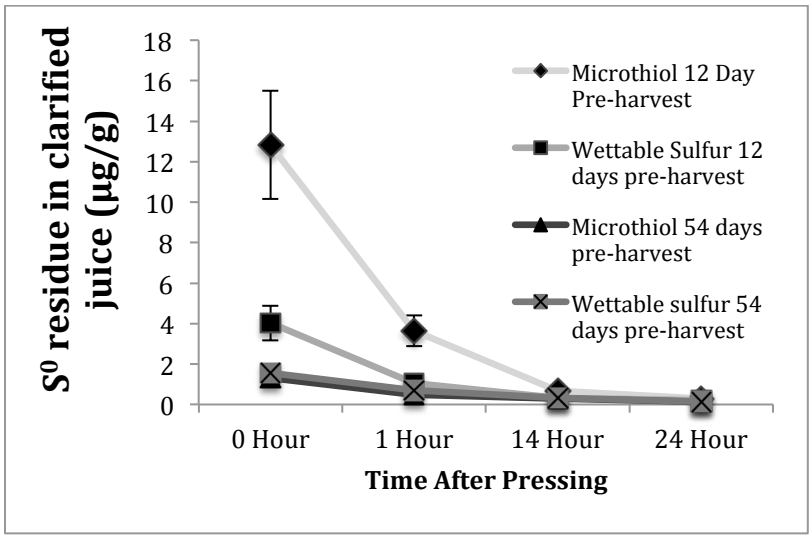

Figure 3.4. Elemental sulfur residue (S^0) present in juice pressed from fruit that received sequential applications of two commercial sulfur formulations (4.48 kg/ha S^0) during the 2011 season, ceasing either 54 or 12 days before harvest. Samples were obtained from 30cm below the juice surface in the carboy using a wine thief, at the post-pressing time intervals indicated.,

Residue fate during vinification and settling. In 2009, there was a dramatic reduction in S^0 residue levels measured on the fruit versus those in the settled must, with must residues approximately 10-25% of those on the fruit and the greatest reductions occurring in treatments with the highest initial concentrations (supplemental Table 3.2). In 2009, H_2S concentration was measured only in the finished wine, and all levels were below 1 µg /l with no significant differences among treatments. In 2010 and 2011, S^0 residue levels were tracked throughout vinification, and H_2S production during fermentation was monitored. In 2010, mean residue levels for all treatments decreased from a range of 4.6-60.8 µg/g on the grapes down to 1.5-15.5 µg/g in the un-clarified must. S^0 residue levels in the juice declined substantially further after settling, to between 0.43 and 1.75 µg/g, leaving residue levels in the settled fraction of 23.9- 174.1 µg/g. The S^0 residue on the grapes correlated well with the residue in un-clarified juice (R^2=0.90, p=0.014). However, S^0 residues on grapes did not correlate with either the levels in the clarified juice (R^2=0.37, p=0.28) or H_2S produced during the fermentation (R^2=0.45, p=0.21). However, S^0 concentrations in the settled must were good predictors of total H_2S production (R^2=0.69, p<0.001).

A similar pattern of the fate of S^0 residue on grapes during fermentation was observed in 2011, with grape residue levels again being a good predictor of residue levels in the unsettled must (R^2=0.74, *p*=0.002). To better control for the degree of must settling, turbidity levels were monitored over 24 hours, and all clarified juice samples were below 20.0NTU (18.2±1.8, no significant differences among treatments [p = 0.05, Tukey's HSD]). Whereas initial S^0 residues in the must ranged from 0.82-18.22µg/g (except undetectable in the control treatment), these declined to only 0.09-0.35 µg/g after settling (Figure 3.4). After settling the only differences observed were that the residue in the 12-day PHI Microthiol treatment was higher than in both the wettable sulfur treatment that ended 25 days preH and the Microthiol treatment ending 54 days prior to harvest (0.29±.09, 0.12±.06, and 0.12±.05µg/g respectively). Total H_2S released during fermentations varied from among all replicates from 2.34-84.24 µg /l, however no differences were observed among any treatments, including the control. In 2011, with S^0 levels ranging from 0.09-0.35µg/g in the clarified must, no relationship between S^0 concentrations a H_2S production was observed. The S^0 residue levels on the grapes, or in the unsettled or settled must were all poor predictors of H_2S for the 2011 fermentations.

Skin contact effect on S^0 persistence and H_2S production. At harvest, Cabernet fruit used in the vinification trials had S^0 residue levels of 11.4µg/g ±1.2. By the time of inoculation, mean must S^0 levels ranged from 0.05-0.20 µg/g in those fermentations that were pressed and settled first, whereas those undergoing a 1- or 2-week maceration exhibited S^0 levels of 10.8 and 11.1µg/g respectively (Table 3.1)**.** The treatments fermented on the skins produced increased levels of H_2S during the course of the fermentations, with mean values for the 1- and 2-week macerations approximately two to three times those of the treatments undergoing settling before inoculation.

Table 3.1. Persistence of S^0 on Cabernet franc grapes, as affected by vinification method

	S^0 content before settling[b]			S^0 content at inoculation[c]			S^0 content at racking[d]			S^0 content in lees[e]			H_2S produced during fermentation		
Treatment[a]	Mean (µg/g)	SD		Mean (µg/g)	SD		Mean (µg/g)	SD		Mean (µg/g)	SD		Mean (ng/ml)	SD	
Whole-cluster pressed	1.24	0.23	b[f]	0.20	0.09	b	nd	-		49.3	5.6	a	70.5	5.1	a
Crushed/ destemmed	0.60	0.01	a	0.05	0.00	a	nd	-		44.0	3.0	a	67.8	3.2	a
24-hour skin contact	1.92	0.16	c	0.18	0.08	b	nd	-		53.6	11.7	a	75.6	8.0	a
1-week maceration	NA	-		10.8	0.8	c	1.2	0.3	a	115.0	6.9	b	140.6	9.4	b
2-week maceration	NA	-		11.1	1.1	c	0.8	0.1	a	163.0	15.4	c	179.2	35.0	b

[a] All grapes for all vinification treatments received an application of Microthiol at 2.69kg/ha, 10 days before harvest, with treatments varying by skin contact during vinification.
[b] Samples for must content before settling were taken immediately after pressing, with no samples included for those treatments fermented on the skins as they did not undergo this step.
[c] Must content at inoculation followed racking of the sediment for those processed as white wines.
[d] Wines were racked 1-week after confirmation that they had fermented to dryness.
[e] Volume of gross lees amounted to roughly 5% of volume before racking.
[f] Different lower-case letters at same date of measurement (a, b, c) indicate difference in means by Tukey HSD at a significance level of *= $p < 0.05$. Tukey HSD analysis was preceded by ANOVA to confirm variables contributed to difference.

Discussion

Effective PM control in areas and seasons with high disease pressure routinely requires >10 fungicide applications per season. A standard fungicide is S^0, applied in either a sprayable formulation or as a dust. S^0 has the benefits over alternatives of being inexpensive, at low risk for pathogen resistance development and relatively environmentally benign. Late season application of S^0 has however, been tied to increased H_2S production during fermentation and reduced aromas in the wine [4-6, 8].

There is disagreement as to what concentration of S^0 residue causes increased H_2S production during fermentation. Concentrations of S^0 ≥10µg/g consistently have been tied to such increases, with some studies finding increases with residues as low as 1µg/g [7, 19]. Others have found that levels as high as 3.2µg/g did not have an effect [20]. Data from 2010 and 2011 suggest that under our vinification parameters, must residue levels somewhere between 0.52-2.16µg/g were necessary to induce increased

H_2S production. However, increased H_2S production during fermentation will not necessarily cause reduced character in the finished wine, as much of the H_2S is lost to volatilization. A better understanding of the fate of H_2S during fermentations is needed to use H_2S production levels as a predictor of the likelihood of fault development.

Earlier research into the impact that elemental S^0 application in the vineyard has on H_2S production in wine has been focused primarily on identifying the residue concentration of S^0 in the must at inoculation that causes an increase. Far less work has gone into understanding how S^0 applied in the vineyard persists on the grapes and when applications should be stopped to avoid excess residue levels in the fermentation. The studies that have been conducted, quantify the S^0 which can be rinsed from intact berries using either a water surfactant mixture [9], or petroleum ether [7]. During method development we found these methods to be inadequate for quantitative removal, instead opting for blended whole cluster samples for our quantification. This may explain why we observed residue levels as high as 86 µg/g of residue on cluster while others reported maximum levels <14 µg/g immediately after S^0 application [9]. Additional research is needed to ascertain whether the increased recovery of whole cluster is due to S^0 adsorbance to the waxy layer of the fruit, or from residue persisting on the rachis being a major contribution to final S^0 concentrations.

Of the limited studies on S^0 persistence, Thomas et al. (1993) determined that S^0 residue levels would not exceed 3.2 µg/g if applications ceased when fruit had matured to above 7° brix. Although fruit are resistant to new infections far before this point of development, control of PM may nevertheless be necessary beyond it as the rachis and new shoot growth remain susceptible until much later [1]. Our research shows that S^0 residue levels can greatly exceed 3.2 µg/g when applications continue to within 35 days of harvest, and that they consistently exceeded 10 µg/g when S^0 is applied within 25 days of harvest (Figure 3.1). In addition to the timing of the final application, S^0 formulation and application rate can also affect residue levels and persistence, both at harvest and throughout the season. For those concerned with residue levels on fruit, it appears that reducing application rates can have a significant impact on reducing final residues, thereby potentially allowing applications later closer to harvest. In 2010,

applications of wettable sulfur at 2.69 kg/ha stopping 22 days preH were comparable to the concentrations observed for treatments stopping 33 days preH when 5.38 kg/ha was applied (Figure 3.1).

While vineyard treatments can have a significant influence on S^0 residue levels getting into the fermentation, they are not necessarily predictive, as vinification decisions may ultimately have the greatest influence. In both 2010 and 2011, residue on grape clusters was a good predictor of residue levels following crushing and destemming but did not correlate well with residue levels post clarification, as S^0 residues in clarified juice were uniformly low regardless of the level beforehand. The influence of variable vinification decisions was further demonstrated by the difference in H_2S produced during fermentations that had received a constant vineyard S^0 treatment. Red wine vinification (fermenting with extended skin contact) produced significantly more H_2S than whites. Though no differences were observed within treatments either vinified as white wines (whole-cluster pressed, crushed/destemmed or 24-hour soak), or red wines (1-week or 2-week maceration). The difference is to be expected given the higher S^0 residue present in the red than the white style fermentations. At the start of the red fermentations nearly all of the 11 μg/g of S^0 initially present on the fruit was present in the must, whereas musts from the various white wine style vinifications retained only 0.2 μg/g or less.

This study did not attempt to decouple the potential reasons for S^0 loss in the vineyard, such as temperature, precipitation, canopy management or variety, and further work is needed to understand what roles these factors play in S^0 accumulation and persistence. With a greater understanding of these factors improved prediction of S^0 residue at harvest may be possible. However, in the meantime monitoring S^0 residue levels with the assay used in this study is a viable option for producers looking to inform their viticultural and vinification decisions relative to this factor.

Conclusion

It is likely S^0 will continue to play an important role in PM control due to its cost and efficacy. Ultimately, decisions in the vineyard and winery as well as uncontrollable factors such as weather will dictate how much of an effect S^0 will have on wine quality. As is often the case with viticultural and winemaking decisions there is not one "right" answer, instead there are a number of acceptable paths. In general, the white wine making process offers more opportunity for the reduction of S^0 residue levels than red wine making. When applying S^0 late in the season the assay utilized in this study offers a method for growers and winemakers to educate their decision and mediate risk of producing wines with reduced aromas.

References

1. Gadoury, D.M.; Cadle-Davidson, L.; Wilcox, W.F.; Dry, I.B.; Seem, R.C.; Milgroom, M.G. Grapevine powdery mildew (Erysiphe necator): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. *Molecular plant pathology* **2011,** *13,* 1-16.

2. Savocchia, S.; Mandel, R.; Crisp, P.; Scott, E.S. Evaluation of 'alternative'materials to sulfur and synthetic fungicides for control of grapevine powdery mildew in a warm climate region of Australia. *Australas. Plant Pathol.* **2011,** *40,* 20-27.

3. Sluiter, E. The production of hydrogen sulphide by animal tissues. *Biochem. J.* **1930,** *24,* 549.

4. Rankine, B.C. Nature, origin and prevention of hydrogen sulphide aroma in wines. *J. Sci. Food Agric.* **1963,** *14,* 79-91.

5. Acree, T.E.; Sonoff, E.P.; Splittstoesser, D.F. Effect of yeast strain and type of sulfur compound on hydrogen sulfide production. *Am. J. Enol. Vitic.* **1972,** *23,* 6.

6. Schutz, M.; Kunkee, R.E. Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast. *Am. J. Enol. Vitic.* **1977,** *28,* 137.

7. Wenzel, K.; Dittrich, H.; Seyffardt, H.; Bohnert, J. Schwefelrückstände auf Trauben und im Most und ihr Einfluss auf die H 2 S-Bildung. *Wein-Wissenschaft* **1980,** *35,* 414-420.

8. Wenzel, K.; Dittrich, H. Zur Beeinflussung der Schwefelwasserstoff-Bildung der Hefe durch Trub, Stickstoffgehalt, molekularen Schwefel und Kupfer bei der Vergärung von Traubenmost. *Wein-Wissenschaft* **1978,** *33,* 200-213.

9. Thomas, C.S.; Gubler, W.D.; Silacci, M.W.; Miller, R. Changes in elemental sulfur residues on Pinot noir and Cabernet Sauvignon grape berries during the growing season. *Am. J. Enol. Vitic.* **1993,** *44,* 205.

10. Kwasniewski, M.T.; Allison, R.B.; Wilcox, W.F.; Sacks, G.L. Convenient, inexpensive quantification of elemental sulfur by simultaneous in situ reduction and colorimetric detection. *Anal. Chim. Acta* **2011,**

11. Merwin, I.A. National Viticulture Research Conference, 13–15 July 2010, Geneva, New York. *Am. J. Enol. Vitic.* **2010,** *61,* 563A.

12. Joseph, C.M.L.; Bisson, L.F.; Sommer, S.; Herr, P.; Wacker, M.; Schmarr, H.G.; Fischer, U.; Favier, M.; Bilhère, E.; Lonvaud-Funel, A. Abstracts from Presentations at the ASEV 62nd National Conference 22–23 June 2011, Monterey, CaliforniaMetabolomic Analysis of Brettanomyces bruxellen. *Am. J. Enol. Vitic.* **2011,** *62,* 386A-411A.

13. Kwasniewski, M.T.; Wilcox, W.F.; Sacks, G.L. The persistence of late season elemental sulfur sprays during berry ripening and vinification, assessed using a simple inexpensive method for quantification. **2012,**

14. He, Z.; Trela, B.; Nisbet, M.; Mansfield, A.K.; Martinson, T.; Sacks, G.; Schmitt, D.M.; Manns, D.C.; Sudano, A.M.; de Orduña, R.M. Abstracts from Presentations at the ASEV Eastern Section 37th Annual Meeting & National Viticulture Research Conference, 16–19 July 2012, Traverse Cit. *Am. J. Enol. Vitic.* **2012,** *63,* 579A-589A.

15. Hamilton, J.; Palmiter, D. *Orchard Tests For Apple Scab in New York State: I. Sulfur Fungicides, Bulletin No. 747.* New York State Agricultural Experiment Station: Geneva, NY, 1951;

16. Park, S.K. Hydrogen Sulfide Detection Tube For Alcoholic Beverages. *Hydrogen sulfide detection tube for alcoholic beverages* **2000,** *09/182,953,*

17. Ugliano, M.; Henschke, P.A. Comparison of three methods for accurate quantification of hydrogen sulfide during fermentation. *Anal. Chim. Acta* **2010,** *660,* 87-91.

18. Park, S.K. Development of a method to measure hydrogen sulfide in wine fermentation. *Journal of microbiology and biotechnology* **2008,** *18,* 1550-1554.

19. Thoukis, G.; Stern, L.A. A Review and some Studies of the Effect of Sulfur on the Formation of Off-Odors in Wine. *Am. J. Enol. Vitic.* **1962,** *13,* 133-140.

20. Thomas, C.S.; Boulton, R.B.; Silacci, M.W.; Gubler, W.D. The effect of elemental sulfur, yeast strain, and fermentation medium on hydrogen sulfide production during fermentation. *Am. J. Enol. Vitic.* **1993,** *44,* 211.

Supporting Information

Table 3.2. Sulfur residue levels on Chardonnay grape clusters taken during the 2009 season

Sulfur residue in Chardonnay must and on Grapes (µg/L)								
			Unsettled must[b]			Fruit		
Treatment number	Microthiol application Rate (kg/ha)	Days before harvest [a]	Mean (µg/g)[c]	SD		Mean (µg/g)	SD	
0	-	Control	0	0	a[d]	0	0	a
1	2.69	68	0.1	0.1	a	0.2	0.2	ab
4	5.38		0	0	a	0.2	0.7	ab
2	2.69	40	0.4	0.1	b	1.5	0.4	b
5	5.38		0.4	0.3	a	1.3	0.7	b
3	2.69	12	6.8	0.7	c	43.4	7.5	c
6	5.38	12	5.2	0.9	c	51.6	8.1	c

[a]A single application of a micronized formulation of S^0 Microthiol Dispers® was made either 12, 40 or 68 days pre-harvest, at a rate of either 2.69 or 5.38 kg/ha.

[b]Sulfur residue levels for "unsettled must" were taken immediately after pressing fruit.

[c]Mean values give are for sulfur residue measured on 5-cluster samples taken from each of the 6 treatment panel replicates.

[d]Means within a row column not followed by a common letter are significantly different (Tukey HSD, *P* <0.05). Tukey analysis was performed following confirmation by 2-way ANOVA that variables contributed to differences at a significant level.

Table 3.3. Sulfur residue levels on Chardonnay grape clusters taken through out the 2010 season

					Sample Date[a]																	
					30-Aug		1-Sep		3-Sep		7-Sep		11-Sep		15-Sep		24-Sep		29-Sep-12		1-Oct	
					Days before harvest																	
					32		30		28		24		20		16		7		2		0	
Treatment number	Last application date[b]	Days before harvest	Formulation	Rate (kg/ha)	Mean (µg/g)[c]	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD
1	12-Aug	50	Microthiol	2.69	3.5 ±	2.0 f[e]	4.0 ±	1.7 d	4.2 ±	1.4 c	3.0 ±	1.0 e	2.8 ±	0.7 d	1.4 ±	0.8 f	0.8 ±	0.5 f	0.3 ±	0.4 f	0.2 ±	0.2 e
3	27-Aug	35	Microthiol	5.38	28.6 ±	4.5 bcd	21.9 ±	3.6 a	17.3 ±	3.3 a	11.8 ±	1.8 ab	8.8 ±	0.9 d	9.3 ±	0.7 de	10.0 ±	3.0 ef	5.1 ±	1.5 ef	4.6 ±	0.5 de
3			Kumulus	5.38	34.9 ±	6.6 ab	20.8 ±	3.7 a	6.9 ±	2.8 bc	8.7 ±	2.9 bcd	9.0 ±	1.1 d	7.9 ±	0.9 def	2.7 ±	0.7 ef	1.5 ±	0.8 f	1.2 ±	0.7 de
4			Kumulus	2.69	20.9 ±	3.1 de	17.5 ±	4.4 ab	7.9 ±	2.6 bc	8.3 ±	2.3 bcd	4.0 ±	1.3 d	3.6 ±	0.6 ef	2.6 ±	0.9 ef	0.7 ±	0.4 f	0.6 ±	0.3 e
5	9-Sep	22	Microthiol	5.38	24.5 ±	2.3 cde	19.9 ±	3.5 a	19.2 ±	1.9 a	15.2 ±	1.1 a	49.5 ±	2.2 b	52.5 ±	5.4 a	24.7 ±	1.4 c	24.2 ±	3.1 c	19.1 ±	3.7 c
6			Kumulus	5.38	30.8 ±	5.0 abc	14.8 ±	2.2 ab	10.4 ±	4.4 b	10.5 ±	2.6 b	54.7 ±	7.0 ab	30.7 ±	5.2 b	20.9 ±	3.2 cd	18.2 ±	4.2 cd	14.3 ±	2.2 c
7			Kumulus	2.69	19.9 ±	2.7 e	6.8 ±	1.6 cd	10.0 ±	3.4 b	5.7 ±	2.0 de	24.5 ±	5.1 c	14.7 ±	3.2 cd	11.5 ±	1.4 de	7.7 ±	1.6 e	6.4 ±	2.6 d
8	23-Sep	8	Microthiol	5.38	28.3 ±	1.8 bcd	21.3 ±	2.0 a	18.7 ±	2.7 a	14.1 ±	1.4 a	49.7 ±	4.5 b	50.2 ±	6.8 a	66.7 ±	7.2 b	48.4 ±	6.6 b	39.0 ±	5.6 a
9			Kumulus	5.38	37.6 ±	7.3 a	15.6 ±	1.7 abc	11.5 ±	2.5 b	9.5 ±	1.3 bc	57.0 ±	4.2 a	35.1 ±	3.9 b	85.9 ±	9.3 a	54.6 ±	3.8 a	27.2 ±	5.6 b
10			Kumulus	2.69	19.6 ±	2.6 e	6.5 ±	1.7 bcd	6.6 ±	1.6 bc	6.6 ±	1.0 cd	20.8 ±	4.5 c	19.5 ±	3.6 c	30.0 ±	7.5 c	17.8 ±	2.6 d	14.3 ±	2.2 c

[a] All treatments were sampled at each sampling date.

[b]Sequential sprays were applied to designated vines on 12 Aug, 27 Aug, 9 Sep, and 23 Sep , with the final application for each treatment as noted . Within a timing regime, treatments varied by S^0 formulation and application rate.

[c]Mean values represent sulfur residue measured on 5-cluster samples taken from each of the 6 replicate treatment panels per treatment.

[d]Means within a column not followed by a common letter are significantly different ($P < 0.05$) according to the Tukey HSD test. Tukey analysis was performed following confirmation by 2-way ANOVA that variables contributed to differences at a significant level.

Table 3.4. Sulfur residue levels on Riesling grape clusters taken through out the 2011 season

						Sample Date															
						15-Aug		24-Aug		30-Aug		6-Sep		15-Sep		22-Sep		29-Sep		9-Oct		16-Oct	
						62		53		47		39 40		31		24		17		9		0	
Treatment number	Last Application Date	Days before harvest	Formulation	Application Rate		Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD	Mean (µg/g)	SD
1	23-Aug	54	Microthiol	4.48kg/ha		13.3±	3.5a	9.4±	1.0c	8.1±	0.5b	5.8±	0.8abc	4.3±	0.8e	6.4±	0.8cd	3.1±	0.5d	4.0±	0.9e	3.7±	0.9e
2			Kumulus	4.48kg/ha		11.3±	2.3a	15.6±	3.2a	6.6±	2.1bc	6.0±	1.5abc	5.4±	0.8de	7.5±	1.7cd	4.5±	0.4d	2.2±	0.6e	1.9±	0.6f
3	8-Sep	38	Microthiol	4.48 and 2.24kg/ha[e]		11.1±	2.4a	8.4±	0.9c	2.4±	0.3d	4.3±	0.6bc	3.8±	1.2e	3.8±	0.5d	2.8±	0.5d	3.9±	0.6e	3.7±	0.6ef
4			Microthiol	4.48kg/ha		11.5±	1.9a	10.3±	1.6c	4.8±	1.3c	5.3±	1.0bc	7.0±	1.4c	5.9±	0.9cd	4.7±	0.5cd	4.1±	0.7e	3.7±	0.7ef
5			Kumulus	4.48kg/ha		11.0±	1.1a	15.0±	a 3.5b	7.0±	0.6bc	7.5±	0.8ab	9.4±	1.0b	8.6±	1.9c	1.7±	0.3d	4.2±	1.1e	4.3±	1.1e
6	21-Sep	25	Microthiol	4.48kg/ha		10.1±	1.8a	10.0±	1.5c	8.5±	1.5ab	4.5±	1.2c	6.1±	1.5cd	22.9±	2.2b	9.3±	2.1b	10.1±	0.9d	7.5±	0.9d
7			Kumulus	4.48kg/ha		11.0±	2.3a	18.0±	1.3a b	10.4±	1.1a	7.6±	1.5ab	13.5±	2.4a	32.1±	3.5a	20.1±	2.0a	13.4±	1.2c	12.7±	1.2c
8	6-Oct	12	Microthiol	4.48kg/ha		10.1±	1.1a	11.4±	0.6c	5.0±	0.6c	4.9±	2.4c	7.7±	0.8c	29.7±	1.3a	8.2±	1.0bc	31.5±	2.7a	24.2±	2.7a
9			Kumulus	4.48kg/ha		10.4±	1.3a	16.0±	2.2a	8.1±	1.5b	8.4±	1.9a	12.1±	1.8a	25.7±	2.7b	20.6±	4.4a	18.3±	2.9b	16.7±	2.9b

[a] All treatments were sampled at each sampling date.

[b]Sequential sprays were applied to designated vines on 13 Jul, 27 Jul, 10 Aug, 23 Aug, 8 Sep, 21 Sep, and 6 Oct with the final application for each treatment as noted. Within a timing regiment, treatments varied by S^0 formulation and application rate.

[c]Mean values represent sulfur residue measured on 5-cluster samples taken from each of the 6 replicate treatment panels per treatment.

[d]Means within a column not followed by a common letter are significantly different ($P < 0.05$) according to the Tukey HSD test. Tukey analysis was performed following confirmation by 2-way ANOVA that variables contributed to differences at a significant level.

[e]The treatment received Microthiol applications at 4.48kg/ha for the firs three applications and 2.24kg/ha on 23 Aug and 8 Sep.

Chapter 4

INVESTIGATIONS INTO THE FATE AND REEMERGENCE OF H_2S IN WINE

Abstract

Volatile Sulfur Compounds (VSCs) are important to wine aroma due to their contribution to varietal character, fermentation-derived aromas and potential for causing consumer rejection at high concentrations due to "reduced" character. A number of factors that affect the formation of VSC have been characterized, yet it is still not possible to predict at the time of bottling whether a wine will develop reductive character during storage. This is because the VSC profile at bottling will not necessarily relate to the VSC profile after storage. Further, while methods such as copper fining or aeration may mitigate reductive aromas at bottling, they may contribute to the development of reduced character during storage. Preliminary efforts were made to develop a method for predicting reductive character development after bottling. Wines from 2010 field trials that had produced different amounts of H_2S during fermentation were monitored for H_2S development post bottling. Despite being bottled with no detectable H_2S (less than 0.1 μg/l), measurable levels were present in many of the wines 3-weeks after bottling. In wines produced from Cabernet franc grapes that had received an application of S^0 10 days before harvest, H_2S concentrations 3-weeks post-bottling correlated well with the H_2S produced during fermentation (r^2=0.543, p-value=0.002). Treatment of these wines at 3-weeks, 6-month and 12-months post-bottling with the reducing agent dithiothreitol (DTT) resulted in the release of H_2S from all wines that received extended skin contact at a rate that correlated with H_2S production. To determine if quinone-thiol adducts could potentially be a source of latent H_2S, a sulfide adduct of 4-methyl-1,2 benzoquinone, was investigated. Following treatment with the reducing agents DTT and tris(2-carboxyethyl)phosphine (TCEP) detectable amounts of H_2S were releasable from the sulfide-quinone adduct. Additional work is needed to characterize the mechanism and relevance of this finding to wine production.

Introduction

The underlying chemistry of reactive sulfur species in wine is one of the least understood areas of wine flavor chemistry despite the great importance it plays regarding wine quality. Many of the important Volatile Sulfur Compounds (VSCs) in wine have sensory thresholds in the range of 1 to 1000 ng/l [1, 2]. These compounds, and their precursors can either be grape or fermentation derived with production and accumulation effected by many viticultural and enological factors [3-5]. VSCs can increase during reductive storage conditions through poorly understood mechanisms. To date most post-fermentation research has focused on changes in sulfur chemistry related to indirect factors such a closures or copper content of the wine rather than underlying mechanisms of VSC formation or their potential precursors [6, 7]. The research that has been conducted on identifying potential pools of reacted VSCs has been predominantly limited to a few model systems, incorporating a few potential reactants [8, 9]. Without a clear understanding of the fate and potential emergence of VSCs post bottling the risk of a consumer purchasing an unacceptable, potentially faulted, wine is increased.

VSCs can generally be divided into two classes, those that are grape derived and those that are fermentation derived. Grape-derived VSCs include potent thiol containing compounds important to varietal character, some of which accumulate as S-cysteine conjugates in the grape [10]. These S-cysteine conjugates are released during fermentation and can contribute to the varietal character of some wines, and can also be lost to due to oxidative reactions, e.g. reactions with quinones [11]. Fermentation derived VSCs include H_2S, CH_3SH, and other low molecular weight sulfur compounds. During fermentation, H_2S is produced as part of amino acid synthesis by yeast [12]. H_2S production during fermentation can be affected by factors such as juice nutrition [13], turbidity [3], yeast strain [3], fermentation temperature [14] as well as S^0 content [3, 15]. H_2S is highly reactive under wine conditions potentially forming other aroma active compounds such as larger thiols and disulfides [16]. In a model wine system, H_2S and thiol products may become bound to phenolic derived quinones [9].

The wine fault of "reduction" is a blanket term used for wines that exhibit undesirable sulfurous aromas. Reduction is one of the most prevalent and economically damaging flaws in wine [17]. While H_2S

is a potential contributor to reductive aromas in wine, there are several other potent aroma compounds that can be derived from H_2S that may result in a wine being considered to suffer from reduction. Low molecular weight mercaptains can be formed in wine through reactions of H_2S with aldehydes or degradation of S containing amino acids post fermentation [18]. These compounds have aroma thresholds around 1μg/l and are less volatile, and therefore less readily lost due to CO_2 entrainment during fermentation, or through aeration [17]. These compounds are highly reactive and can readily create disulfides in the presence of oxygen. While disulfides are not as aroma active as mercaptans or H_2S, with thresholds in the 10-50 μg/l, they are claimed to pose a greater problem to winemakers as they cannot be removed through the use of copper sulfate [19].

Closures with less oxygen ingress have been observed to cause increased incidence of reduced wines. Screw top closures are a prime example of this effect and their adoption has been suggested as one of the reasons reduced wines are so prevalent today [6]. Additionally, other factors such as glutathione and copper content at bottling can effect reduced sulfur aromas with glutathione addition increasing H_2S and methanethiol during storage [7]. Surprisingly, copper, which is commonly used to remove H_2S, has been shown to increase H_2S content under certain conditions [7]. A wine with both high glutathione content and copper was shown to increase H_2S content from >1 μg/l at bottling to an supra-threshold level of 5 μg/l , 6-months post bottling [7]. This study gives credence to the observations by winemakers that wines will develop reduced character post bottling despite (or perhaps due to) efforts to minimize reduced character at bottling using treatments such as aeration, sulfite addition and copper fining. It has been suggested that disulfide may serve as may be reduced to mercaptans during anaerobic bottle storage, [8] However, under wine conditions, versus a model system, there is question as to whether the conversion of disulfide to thiol occurs [20]. Another possibility is that sulfur-containing amino acid degradation causes the release of thiols [21]. A positive relationship between high amounts of cysteine and glutathione at bottling and increased H_2S has been observed [7]. However, it has yet to be demonstrated that these compounds contribute directly to thiol production rather than acting as protective compounds that prevent oxidation of other thiols.

The lack of knowledge on VSC chemistry between the period following fermentation until a consumer opens a bottle of wine makes it challenging for winemakers to predict a wine's behavior during storage. To properly predict how VSCs produced during fermentation will affect wine flavor at bottle opening it is necessary to understand what reactions they are likely to undergo in the conditions found in wine, what volatile and non-volatile compounds they may react with, and if these reactions may be reversible.

To investigate the relationship between fermentation production of H_2S and emergence post bottling a preliminary study was conducted using wine from a 2010 field study in which elemental sulfur was applied to grapes close to harvest. Additionally, efforts were made to develop a method for predicting a wine's likelihood of developing reductive VSCs during storage. Using this method a sulfide adduct of 4-methyl-1,2 benzoquinone was investigated as a potential source of H_2S post bottling.

Materials and Methods

Chemicals - Cysteine, glutathione, mercaptoethanol, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), sodium sulfide, potassium metabisulfite and polyethylene glycol 400 (PEG 400) were all purchased at ≥99% purity (Fischer Scientific). Alka-Seltzer tablets (Bayer Healthcare, Morristown, NJ) were purchased locally. Ultra high purity nitrogen gas was used (Airgas, Ithaca, NY). Distilled de-ionized water was used for all experiments. Gastec 4LT, 4LL and 4H (Nextteq, Tampa, FL) H_2S detection tubes were utilized. The detection tubes rely on a colorimetric reaction within the tube between evolved H_2S and a metal salt, either mercury chloride (Gastec 4LT detection tube) or lead acetate (all other detection tubes) adhered to a proprietary, inert matrix. The length of the tube darkened is linearly proportional to the quantity of H_2S evolved. Calibrations for the tube ranges used are contained in Chapter 2.

A sulfide adduct of 4-methyl-1,2 benzoquinone was donated by the Waterhouse lab at U.C. Davis. This was produced by reacting an excess of sodium sulfide with 4-methyl-1,2 benzoquinone in an aqueous solution and dried under vacuum. The supplied compound contained unreacted 4-methyl-1,2

benzoquinone, as well as at least four oligomers as determined by the Waterhouse lab by reverse phase LC-MS.

Protocol for Quantification of Free and Latent H_2S. H_2S was quantified using the method discussed in Chapter 2, in which a commercially available H_2S detection tube was attached to a jar and the metal salt in the tube reacted colorimetricly with a linear response. When measuring H_2S the jars were first sparged with N_2 gas to minimize oxidation of H_2S in the sample. When measuring releasable H_2S an alka-seltzer tablet was added prior to testing to remove any free H_2S in the wine. A variation on this method was used to quantify and confirm complete removal of any free H_2S present at bottling. Carboys were fitted with stoppers with two holes and tubing running through each. The wine was sparged with N_2 gas, which was pushed through an aquarium aeration stone. Gas exited though a second tube that ran through the stopper and was fitted with a detection tube at the end. Wines were sparged until no change was observed on the detection tube, in wine with higher H_2S levels this could take over 2 hours.

Evaluation of reducing agents for quantifying latent H_2S. Reducing agents tested included cysteine, glutathione, ascorbic acid, sulfur dioxide, DTT and TCEP. Reducing agents were added to samples buffered to pH 6 with Alka-Seltzer and then left to react for 10 minutes prior to addition of a second and third Alka-Seltzer in sequence, as in the method described in Chapter 2. The concentration of H_2S in wines 21 days after bottling was quantified in Chardonnay and Cabernet franc wines from the 2010 trials. Following confirmation of H_2S development in wines which had contained no measurable H_2S at bottling efforts were made to evolve additional H_2S in these wines through reaction with reducing agents. To assess if the H_2S produced upon reaction with the reducing agent could be the result of residual S^0, "latent" (reducing agent releasable) H_2S was measured in samples with and with out the dispersion step needed for quantitative conversion of S^0 to H_2S, as well as using reducing agents other than DTT that had been less effective at conversion of S^0 to H_2S. Additional measurements were taken from Cabernet Franc

wines for H_2S and latent H_2S concentrations using TCEP as the reducing agent at six and twelve months post-bottling.

Free and Latent H_2S in Wines Produced with S^0 Treated Grapes. Wines produced from 2010 field studies investigating elemental sulfur persistence in the vineyard and during vinification were used in this analysis. Vinification and treatment conditions for these wines are discussed in depth in Chapter 3. Wines, either Cabernet Franc or Chardonnay, from two experiments were used. The Cabernet Franc grapes had received an application of a sulfur containing fungicide (Microthiol) 10-days before harvest at a rate of 2.69 kg/ha. Treatments included grapes either being 1) whole cluster pressed 2) crushed/destemmed and pressed 3) crushed/destemmed, cold-soaked for 24 hours then pressed 4) crushed/destemmed, macerated with skins for 7 days, then pressed or 5) crushed/destemmed, macerated with skins for 14 days, then pressed. Chardonnay grapes were vinified the same but received different sulfur application in the vineyard (explained in detail in Chapter 3).

The concentrations of free and latent (DTT releasable) H_2S were measured for the Cabernet Franc wines at 21-days, 6-months and 12-months post bottling. The concentrations of H_2S and latent H_2S was measured for the Chardonnay wines only at the 21-day post bottling time point. At bottling the Cabernet franc and Chardonnay wines had no quantifiable levels of H_2S, having been sparged with N2 gas.

Evaluation of 4-methylcatechol sulfide adducts as precursor of latent H_2S. An adduct of 4-methylcatechol and H_2S was supplied from the Waterhouse lab (U.C. Davis). The product analyzed in the Waterhouse lab using LC/MS contained at least four of oligomers of the adduct in addition to unreacted 4-methyl catechol. Solutions of the adduct where treated with reducing agents, as described above for the wines, to assess the reversibility of the reaction that formed the sulfide quinone adducts. Following positive results, efforts were made to separate the oligomers, and unreacted 4-methycatechol using thin layer chromatography and liquid/liquid extraction. Separation of four distinct bands visible following

iodine development using a solvent system of 30% by volume mixture of ethyl acetate and hexane was carried out. These compounds were tested individually for their ability to form H_2S.

Statistics. SAS .JMP version 9.0.2 was used for statistical analysis (SAS Cary, NC). Parametric mean testing was done using Tukey HSD, following confirmation by ANOVA of differences.

Results and Discussion

Evidence of latent H_2S pool and development of protocol for quantification. Informal sensory observation indicated reductive character 3-weeks post bottling in some of the wines produced from the 2010 field trials discussed in Chapter 3, in spite of all wines having been sparged with N_2 at bottling until no measurable H_2S remained. This observation prompted further investigation of the re-emergence of H_2S in bottled wines, and the potential precursors contributing to this pool of H_2S. While one potential reason for this reemergence could be the persistence of S^0 residues in the wine through bottling, experiments that will be detailed further below suggest compound(s) other than S^0 contribute to this pool.

Wines from the 2010 field trial were initially tested using the method outlined in chapter 2 for orthorhombic S^0 quantification. While this process produced positive results for evolving additional H_2S other reducing agent were also used to rule contribution of H_2S from other compounds such as di-sulfides. As well as to investigate the potential for producing a protocol based of sensory evaluation in changes following a simulated aging of the wine using a reducing agent rather than relying on H_2S quantification. DTT having a strong thiol-like aroma itself would not be an option in this sort of evaluation. Ascorbate, which has been suggested as a chemical capable of breaking disulfide bonds to produce free thiols was ineffective at producing additional H_2S, or a change in aroma [19]. Glutathione and SO_2 were also ineffective under test conditions in evolving H_2S. However, substitution of TCEP for DTT not only evolved comparable amount of H_2S from several of the wines but also resulted in a detectable 'reduced' aroma. While TCEP was found in Chapter 2 to sparingly convert S^0 to H_2S, it caused less than $1/10^{th}$ the

conversion possible with DTT. Also, during the development of the S^0 assay in Chapter 2 it was found that quantitative reduction of orthorhombic S^0 to H_2S only occurred when the sample first underwent a dispersion step in 4x the sample's weight of PEG 400 in a 80°C water-bath for at least 5 minutes. This dispersion step resulted in a roughly 500% increase in the conversion of S^0 to H_2S. The yield of H_2S from the latent pool of H_2S contained in these wines was unaffected when the dispersion step was omitted. Taken together, the observation that TCEP was efficient at H_2S production (with or with out sample dispersion in PEG), and that PEG was unnecessary to improve yields led to the conclusion that the compounds contributing to the latent pool of H_2S in these wines was not orthorhombic S^0.

Free and Latent H_2S in Wines Produced with S^0 Treated Grapes. Free and latent (DTT-releasable) H_2S were measured at three different time points, 3-weeks, 6-months and 12-months post bottling and at 3-weeks after bottling for Chardonnay. Only one time point was measured for Chardonnay wines due sample limitations, as only approximately 700ml of each replicate was produced and bottled (into 187ml bottles). Cabernet Franc wines made from grapes, which had received a late season application, produced more H_2S with increased skin contact (Table 4.1). While H_2S levels at bottling correlated poorly with H_2S produced during fermentation, H_2S measured 3-weeks and 6-months post bottling correlated well with H_2S production during fermentation (Table 4.3). No detectable H_2S existed 12-months post-bottling in any of the Cabernet Franc wines (>0.1 µg/l). A large pool of latent H_2S was observed in wine that had received skin contact, with some producing more than 80 µg/l. At these concentration there is potential for them to play an important role in a wines aroma profile, if released during bottle storage. In cabernet franc the latent H_2S at all time points correlated well with H_2S production during fermentation.

Table 4.1. H_2S concentrations produced during fermentation and in wine for 2010 Cabernet Franc wines.

	At Bottling[a]					21-Days Post Bottling					6-month Post Bottling					1-year Post Bottling				
Treatment[b]	H_2S Produced	SD		H_2S at Bottling	SD	Free	SD		Latent[c]	SD	Free	SD		Latent	SD	Free	SD		Latent	SD
whole cluster	70.5	5.1	b	nd	-	0.4	0.1	c	0.1	0.2 d	nd	-		nd	-	nd	-		nd	-
crushed-destemmed	67.8	3.2	b	nd	-	0.6	0.0	bc	0.1	0.2 d	nd	-		nd	-	nd	-		nd	-
cold soak	76.0	2.9	b	8.4	0.3 a[d]	1.1	0.1	a	17.6	1.7 c	nd	-		10.0	2.0 c	nd	-		10.3	3.5 bc
1-week maceration	140.7	9.5	a	1.6	2.7 bc	1.0	0.3	ab	38.6	5.0 b	1.7	0.4	a	30.7	4.7 b	nd	-		28.9	8.5 ab
2-week maceration	179.3	35.2	a	4.6	2.5 ab	1.4	0.2	a	88.9	6.6 a	5.2	1.2	b	72.0	11.1 a	nd	-		49.7	15.3 a

[a]Timings refer to the period at which H_2S levels were measured.
[b] Grapes for all fermentations received and application of Microthiol 10 days before harvest. Treatments varied by skin contact during vinification
[c] "Latent" refers to H_2S levels releasable when wine was treated with the reducing agent DTT.
[d] Different lowercase letter within a column denote difference between means at a significance level $p<0.05$, compared by Tukey HSD.

Table 4.2. H_2S concentrations produced during fermentation and in wine for 2010 Chardonnay wines.

Formulation and Rate[a]	Spray Timing Before Harvest[b]	H_2S during Fermentation	SD	H_2S At Bottling	H_2S 3-Weeks Post-Bottling	SD	Latent[c]	SD
Wettable Sulfur (2.69kg/ha)	2-weeks	6.3	3.5	nd	0.4	0.1	5.1	0.4
Wettable Sulfur (5.38kg/ha)	2-weeks	26.7	14.6	nd	1.3	0.6	4.8	0.1
Microthiol (5.38kg/ ha)	2-weeks	60.8	11.1	nd	0.4	0.1	6.2	0.5
Microthiol (5.38kg/ ha)	4-weeks	28.3	11.7	nd	0.4	0.1	3.9	0.2
Microthiol (5.38kg/ ha)	6-weeks	11.3	1.5	nd	0.3	0.2	0.6	0.2

[a] Wines were produce from grapes that had received different elemental sulfur fungicide application regimes in the vineyard. With treatments varying by formulation, application rate and the timing of the last application of sulfur.
[b] Applications of elemental sulfur continued until the indicated timing before harvest.
[c] "Latent" refers to H_2S levels releasable when wine was treated with the reducing agent DTT.

A less clear relationship between H_2S production during fermentation and free, or latent H_2S in bottle was observed in Chardonnay wines where vinification practices remained constant throughout treatments, with only S^0 content and formulation varying. However, re-emergence of H_2S and latent H_2S were observed in many of the Chardonnay wines post bottling (Table 4.2). H_2S concentrations, and latent H_2S in Chardonnay did not correlate with H_2S produced during fermentation (data not shown). Latent H_2S

in Chardonnay was roughly similar to the similarly processed Cabernet Franc wines, with Cabernet Franc wines fermented on the skins exhibiting latent H_2S levels over an order of magnitude higher.

However, limited conclusions can be drawn from these observations without further investigation as this experiment did not control for the fact that, in addition to varying levels of S^0 in the fermentations, wines also would be expected to have varying amounts of phenolic compounds due to their increased skin contact. Still, these results suggest that there may be a pool of H_2S potentially releasable under reductive conditions that is higher in red wines versus whites. H_2S is known to react with polyphenol quinones, so one explanation for higher latent H_2S in red wines [9].

Table 4.3. Regression of H_2S produced during Cabernet Franc fermentations, by H_2S levels measured in the wines.

Treatment[ab]	Regression Line	r^2	p-value
H_2S at Bottling	=1.15+0.014x	0.33	0.514
Free H_2S 21-days post bottling	=0.25+0.006x	0.54	0.002
Latent H_2S 21-days Post bottling	=-41.49+0.661x	0.88	<0.0001
Free H_2S 6-months post bottling	=-2.60+0.037x	0.69	0.0001
Latent H_2S 6-months Post bottling	=-36.86+0.556x	0.91	<0.0001
Free H_2S 12-months post bottling[c]	-	-	-
Latent H_2S 12-months Post bottling	=-25.77+0.409x	0.91	<0.0001

[a] Concentrations of H_2S either measured existing in a free form at pH6, or releasable upon reaction with the reducing agent DTT (latent H_2S).
[b] Grapes for all vinification treatments received an application of Microthiol at 2.69kg/ha, with treatments varying by skin contact during vinification. This variation in skin contact however also resulted in different amounts of S^0 persisting into the fermentation..
[c] No detectable free H2S existed in any sample 12 months after bottling.

Evaluation of 4-methylcatechol – H_2S adducts as latent precursors of free H_2S. One of the phenolic sulfide adducts observed to exist in wine was donated by the Waterhouse lab, U.C. Davis, to test whether it could produce H_2S under reducing conditions. It was found after initial screening of the adducts

provided, that in addition to the impurities observed by the Waterhouse lab following LC-MS analysis, it appeared that unreacted sodium sulfide was still present at a level of approximately 10%w/w. This was determined by the amount of sulfide quantified when measured by detection tube, without the addition of a reducing agent. Alternatively the 4-methyl-1,2 benzoquinone may exist in a equilibrium at pH6 with a portion of the sulfide being free. Addition of DTT or TCEP prior to H_2S measurement resulted in increased H2S being evolved with recovery of 20%w/w of the sulfide bye weight of adduct added. Reaction with glutathione, ascorbic acid, or sulfur dioxide did not improve recovery of H_2S. Estimating what percent of H_2S was released was not possible with out first better characterization and purification of the adduct mixture as any number of 1,2 benzoquinone oligomers, with or with out sulfide adducts could have been produced in the reaction. Preliminary efforts at separation using thin layer chromatography were successful at separating four different compounds. All separated compounds tested positive for latent H_2S (and not free H_2S) , with the exception of the spot with the lowest Rf value. While these preliminary results indicate the potential release of H2S from the 4-methyl-1,2 benzoquinone adduct, the measurements and reaction took place at pH 6, well above normal wine pH. Further purification is needed to accurately characterize what role 4-methyl-1,2 benzoquinone sulfide adducts may play in wine aroma chemistry.

Conclusions and Future Work

This work provides evidence to support the anecdotal observation common among winemakers that, "stinky fermentations produces stinky wines" despite efforts to remediate the problem. H_2S production during fermentation correlated well with H_2S reemerging weeks post bottling. Also, a pool of "latent" H_2S, which could form free H_2S following treatment with a reducing agent, also correlated well with H_2S produced during fermentation and informal sensory analysis. Using a reducing agent to simulate aging shows promise both for screening wines for their propensity to produce reductive aromas post-bottling as well as characterizing potential chemicals contributing to the latent H_2S pool. In our preliminary investigations it appears that quinone-sulfide adducts, such as the 4-methyl-1,2 benzoquinone

adduct tested, may be a contributor to the latent H_2S pool. However more work is needed to characterize these reactions under wine conditions using more purified chemical standards.

Ultimately the observations outlined in this chapter result in more questions that answers, with several potential avenues for further research. Further work is needed to understand what mechanism(s) cause H_2S produced during fermentation to affect H_2S production after bottling. A number of studies have looked at factors affecting VSC development, such as O_2 levels at bottling, closure choice and sulfur dioxide and copper concentrations, but the latent precursors of H_2S formation in bottle are not well defined. As a first step to unlocking this pathway it may be easiest to first investigate fate of S^0, as well as any products generated throughout the vinification process. S^0 addition allows for an easy and relatively inexpensive method for adding a stable S^0 isotope, the products of which can be tracked throughout vinification. However, it is still unknown whether the increased H_2S observed with S^0 addition to a fermentation is a result of abiotic reduction of the S^0 to H_2S, enzymatic reduction within the yeast cell, or as a stress response from the yeast due to the fungicidal activity of S^0. Addition of ^{34}S to a series of fermentations will make it possible to better determine which of these mechanisms is at play. Additionally, if the S^0 is reacting during the fermentation to produce H_2S, rather than H_2S produced from other sources as a stress response, the products can be further tracked gaining some incite into whether this reaction is biotically mediated by the ratio of S_{32} to S_{34} in the products. Ultimately accurate mass LC-MS maybe the best path for identifying and tracking sulfur contain compounds through fermentation and storage

Use of reducing agents to predetermine a wine's potential for reductive aroma development. In the immediate future there is evidence that, it least in some cases, a wine's likelihood of developing off aromas maybe predicted at bottling through reaction with a reducing agent. Currently however there is no validated method for predicting if a wine will form reductive off-aromas during storage. Two possible forms for this assay are either 1) quantification of H_2S evolved as was 2) use of a non-volatile (or non-aroma active) compound as a reducing agent, which allows for any change to be observed simply by smelling the sample. Each has potential drawbacks, the first limits identification of only increased H_2S

following treatment with reducing agent, the second in non-quantitative. The limitation of the first assay could be overcome if quantification was done using an alternative method such as GC-MS, or GC coupled with a sulfur sensitive detector, however that would be only appropriate for largest wineries with well equipped labs, or for research.

References

1. Tominaga, T.; Furrer, A.; Henry, R.; Dubourdieu, D. Identification of new volatile thiols in the aroma of Vitis vinifera L. var. Sauvignon blanc wines. *Flavour Fragrance J.* **1998,** *13,* 159-162.

2. Siebert, T.; Bramley, B.; Solomon, M. Hydrogen sulfide: Aroma detection threshold study in white and red wines. *AWRI Tech.Rev* **2009,** *183,* 14-16.

3. Rankine, B.C. Nature, origin and prevention of hydrogen sulphide aroma in wines. *J. Sci. Food Agric.* **1963,** *14,* 79-91.

4. Tominaga, T.; Baltenweck-Guyot, R.; Des Gachons, C.P.; Dubourdieu, D. Contribution of volatile thiols to the aromas of white wines made from several Vitis vinifera grape varieties. *Am. J. Enol. Vitic.* **2000,** *51,* 178-181.

5. Nedjma, M.; Hoffmann, N. Hydrogen Sulfide Reactivity with Thiols in the Presence of Copper (II) in Hydroalcoholic Solutions or Cognac Brandies: Formation of Symmetrical and Unsymmetrical Dialkyl Trisulfides. *J. Agric. Food Chem.* **1996,** *44,* 3935-3938.

6. Lopes, P.; Silva, M.A.; Pons, A.; Tominaga, T.; Lavigne, V.; Saucier, C.; Darriet, P.; Teissedre, P.L.; Dubourdieu, D. Impact of oxygen dissolved at bottling and transmitted through closures on the composition and sensory properties of a Sauvignon Blanc wine during bottle storage. *J. Agric. Food Chem.* **2009,** *57,* 10261-10270.

7. Ugliano, M.; Kwiatkowski, M.; Vidal, S.; Capone, D.; Siebert, T.; Dieval, J.B.; Aagaard, O.; Waters, E.J. Evolution of 3-mercaptohexanol, hydrogen sulfide, and methyl mercaptan during bottle storage of Sauvignon blanc wines. Effect of glutathione, copper, oxygen exposure, and closure-derived oxygen. *J. Agric. Food Chem.* **2011,**

8. Bobet, R.A.; Noble, A.C.; Boulton, R.B. Kinetics of the ethanethiol and diethyl disulfide interconversion in wine-like solutions. *J. Agric. Food Chem.* **1990,** *38,* 449-452.

9. Nikolantonaki, M.; Jourdes, M.; Shinoda, K.; Teissedre, P.L.; Quideau, S.; Darriet, P. Identification of Adducts between an Odoriferous Volatile Thiol and Oxidized Grape Phenolic Compounds: Kinetic Study of Adduct Formation under Chemical and Enzymatic Oxidation Conditions. *J. Agric. Food Chem.* **2012,** *60,* 2647-2656.

10. Tominaga, T.; des Gachons, C.P.; Dubourdieu, D. A New Type of Flavor Precursors in Vitis v inifera L. cv. Sauvignon Blanc: S-Cysteine Conjugates. *J. Agric. Food Chem.* **1998,** *46,* 5215-5219.

11. Nikolantonaki, M.; Chichuc, I.; Teissedre, P.L.; Darriet, P. Reactivity of volatile thiols with polyphenols in a wine-model medium: Impact of oxygen, iron, and sulfur dioxide. *Anal. Chim. Acta* **2010,** *660,* 102-109.

12. Jiranek, V.; Langridge, P.; Henschke, P. Regulation of hydrogen sulfide liberation in wine-producing Saccharomyces cerevisiae strains by assimilable nitrogen. *Appl. Environ. Microbiol.* **1995,** *61,* 461.

13. Ugliano, M.; Fedrizzi, B.; Siebert, T.; Travis, B.; Magno, F.; Versini, G.; Henschke, P.A. Effect of nitrogen supplementation and Saccharomyces species on hydrogen sulfide and other volatile sulfur compounds in Shiraz fermentation and wine. *J. Agric. Food Chem.* **2009,** *57,* 4948-4955.

14. Schutz, M.; Kunkee, R.E. Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast. *Am. J. Enol. Vitic.* **1977,** *28,* 137.

15. Wenzel, K.; Dittrich, H. Zur Beeinflussung der Schwefelwasserstoff-Bildung der Hefe durch Trub, Stickstoffgehalt, molekularen Schwefel und Kupfer bei der Vergärung von Traubenmost. *Wein-Wissenschaft* **1978,** *33,* 200-213.

16. Thoukis, G.; Stern, L.A. A Review and some Studies of the Effect of Sulfur on the Formation of Off-Odors in Wine. *Am. J. Enol. Vitic.* **1962,** *13,* 133-140.

17. Siebert, T.E.; Solomon, M.R.; Pollnitz, A.P.; Jeffery, D.W. Selective determination of volatile sulfur compounds in wine by gas chromatography with sulfur chemiluminescence detection. *J. Agric. Food Chem.* **2010,**

18. Rauhut, D. Yeasts—production of sulfur compounds. *Wine microbiology and biotechnology.Harwood Academic Publishers, Chur, Switzerland* **1993,** 183-223.

19. Zoecklein, B.W.; Fugelsang, K.C.; Gump, B.H.; Nury, F.S. *Wine analysis and production.* Chapman & Hall New York: 1995;

20. Majcenovic, A.B.; Schneider, R.; Lepoutre, J.P.; Lempereur, V.; Baumes, R. Synthesis and stable isotope dilution assay of ethanethiol and diethyl disulfide in wine using solid phase microextraction. Effect of aging on their levels in wine. *J. Agric. Food Chem.* **2002,** *50,* 6653-6658.

21. Pripis-Nicolau, L.; de Revel, G.; Bertrand, A.; Maujean, A. Formation of flavor components by the reaction of amino acid and carbonyl compounds in mild conditions. *J. Agric. Food Chem.* **2000,** *48,* 3761-3766.

Chapter 5

RELATIONSHIP BETWEEN C_{13} NORISOPRENOID AND CAROTENOID CONCENTRATIONS IN RIESLING AS AFFECTED BY LEAF REMOVAL TIMING

Abstract

Sunlight exposure of winegrape clusters is frequently reported to increase C_{13}-norisoprenoids in resulting wines, but the timing and mechanism of this influence is not well understood. Fruit zone leaf removal was applied to *V. vinifera* cv. Riesling at three timings: 2-, 33- and 68-days past berry set (PBS), and compared to an untreated control. Free and total 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), vitispirane and β-damascenone were measured in juice and wines, and carotenoid profiles were determined in grapes at mid-season and maturity. Significantly higher total TDN was observed in grapes from the 33-days PBS treatment compared to the control and other treatments (195 µg/L vs. 54-87 µg/L). Total vitispirane in juice was also significantly increased in the 33-days PBS treatment, while total β-damascenone was reduced in the 68-days PBS treatment compared to the control. Existing HPLC protocols were modified to allow for quantification of zeaxanthin in *V. vinifera* berries, and zeaxanthin was determined to be significantly higher in the 33-days PBS treatment than the control or other treatments ($p<0.05$). Total TDN in juice correlated with free TDN in wine, with 11.0% ± 2.5% of total juice TDN converted to free TDN in wine. In contrast, total vitispirane increased significantly during fermentation, and was not correlated with vitispirane in juice. In summary, leaf removal at 33-days PBS significantly increased zeaxanthin in Riesling grapes mid-season, total TDN and vitispirane in the juice of mature Riesling grapes, and free and total TDN in finished wine, while earlier or later leaf removal had no effect.

Introduction

The C_{13}-norisoprenoids are one of several classes of grape-derived odor-active compounds associated with wine aroma quality [2]. While trace levels of free C_{13}-norisoprenoids are detectable in juice, the majority of C_{13}-norisoprenoids in wine appear to derive from precursors, including non-volatile C_{13}-norisoprenoids glycosides derived from carotenoid cleavage [50], and can be released during winemaking or storage by enzymatic and non-enzymatic mechanisms [51]. The best studied C_{13}-norisoprenoid in wine and grapes is arguably TDN (l,l,6-trimethyl-1,2-dihydronaphthalene), which is associated with "kerosene" or "petrol" aromas and has an orthonasal sensory threshold of 20 μg/L in wine [8]. TDN has been detected in several varietal wines, but its presence is most closely associated with the aroma of bottle-aged Riesling [52]. While TDN concentrations around sensory threshold are generally acceptable to consumers, excessive levels are considered undesirable, especially in young Riesling [52].

Free TDN in Riesling juice is generally below detection threshold, but TDN concentrations in excess of 200 μg/L in Riesling wine are reported to occur following prolonged storage [8, 53]. TDN precursors, e.g. C_{13}-norisoprenoid glycosides, have been reported in grapes, and the concentration of TDN in a finished wine is proportional to the concentration of acid-releasable TDN precursors in must [2, 52] Warmer growing conditions and greater cluster exposure to sunlight are associated with higher TDN concentrations in finished wines, due to a larger concentration of precursors in the juice [52]. Conversely, lower TDN concentrations in wine are associated with shaded fruit, either through direct means like canopy management [10] and indirectly through increased vine fertilization [54] or irrigation [55] resulting in increased vine canopy. A similar decrease in the concentration of several other volatile C_{13}-norisoprenoid precursors has been observed in shaded clusters, including vitispirane and the actinidols [56]. One possible exception to this trend is β-damascenone, which has been implicated in enhancing fruity aromas in wines. Some authors have reported an increase in β-damascenone in response to cluster shading [10, 52], while others have reported either no change or a decrease in shaded grapes [9, 57].

Because of the clear link between TDN precursor production and cluster light exposure, and assuming lower TDN concentrations were desirable, a superficially obvious solution to reducing the TDN potential of Riesling or other winegrapes would be to avoid cluster exposure. However, increasing berry sun exposure is often desirable for reducing disease pressure [58], decreasing titratable acidity, and potentially for increasing production of other desirable compounds like monoterpenes [59]. Therefore, it is advantageous to identify canopy management practices that will produce desirable outcomes independently of C_{13}-norisoprenoid concentrations, especially TDN. A better understanding of the key period(s) during the growing season in which cluster sun exposure increases C_{13}-norisoprenoid precursors could assist winegrape growers in making appropriate canopy management decisions for targeting specific wine flavor profiles.

The (bio-)chemical mechanisms underlying C_{13}-norisoprenoid precursor formation in grapes have been subject to considerable study [2, 60]. TDN and other C_{13}-norisoprenoids show structural similarities to carotenoids, and there is strong evidence that C_{13}-norisoprenoid precursors in mature grapes are derived via oxidative degradation of carotenoids [61]. The major carotenoids in grapes, β-carotene and lutein, begin to decrease at or just prior to veraison [62]. C_{13}-norisoprenoid precursor formation commences within 1-2 weeks after veraison and may reach a maximum within 30-days post-veraison, although some studies report a late spike in concentration near maturity [52, 61]. Grape C_{13}-norisoprenoids were originally proposed to be formed by abiotic carotenoid degradation, e.g., TDN can be formed from lutein under acidic conditions [63]. Alternatively, a family of carotenoid cleavage dioxygenase (CCD) enzymes has been implicated in production of plant apocarotenoids, e.g. C_{13}-norisoprenoids [64], and a CCD capable of producing C_{13}-norisoprenoids from lutein and zeaxanthin (*VvCCD1*) was recently cloned from grapes [50]. Expression of *VvCCD1* increases at veraison, although a 1-2 week lag is reported to occur between increased transcript expression and a significant increase in glycosylated C_{13}-norisoprenoids. Following enzymatic or non-enzymatic biogenesis, part of the pool of C_{13}-norisoprenoids is proposed to undergo *in vivo* glycosylation, potentially after further transformations (e.g. hydration, oxidation) within the grape berry [60, 65] (Figure 5.1). Grape-derived C_{13}-norisoprenoid glycosides can be hydrolyzed during

fermentation and storage, and both native and glycoside-derived C_{13}-norisoprenoid aglycones can be further transformed enzymatically or non-enzymatically to odor active forms, e.g. TDN and β-damascenone [66, 67].

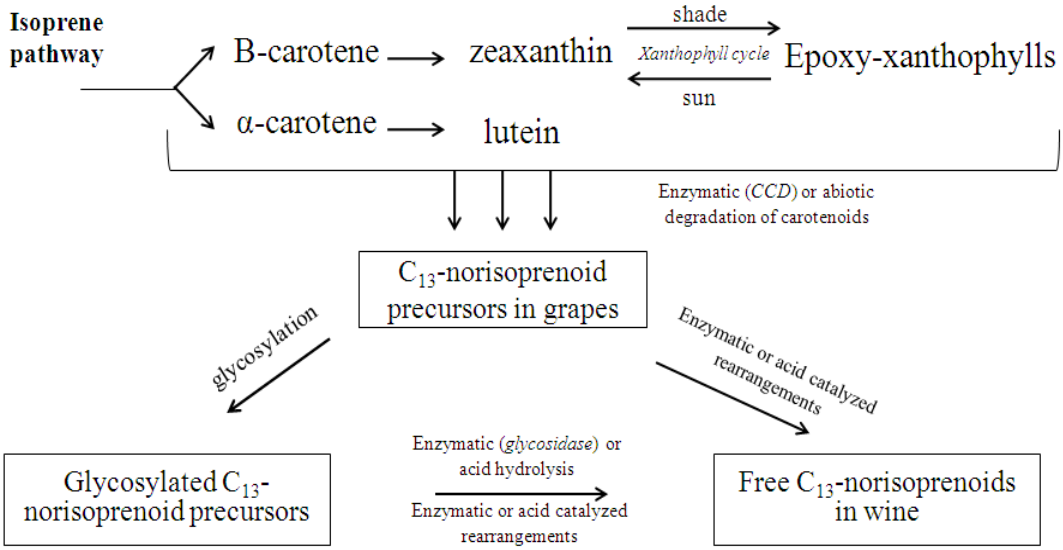

Figure 5.1. (Top) Simplified carotenoid biosynthetic pathway in flowering plants [60]. (Bottom) General mechanism by which norisoprenoid aroma compounds are formed from carotenoids in grapes and wine [68].

Carotenoids are expressed in photosynthetically active tissues of plants as part of Photosystem II (PSII). The major carotenoid species in grapes (β-carotene, lutein) act as light harvesting antennae pigments, while other oxygenated carotenoid species (e.g. neoxanthin, zeaxanthin) participate in photo-protection of the plant via the xanthophyll cycle [68]. Total carotenoid concentrations are believed to be primarily developmentally regulated [68], but environmental factors such as cluster light exposure also influence concentrations [69-71]. Since pre-veraison berries are photosynthetically active, higher concentrations of carotenoids, and thus higher substrate availability, are one potential explanation for higher concentrations of C_{13}-norisoprenoid precursors in sun-exposed grapes [69]. However, cluster exposure does not consistently yield higher concentrations of carotenoids pre-veraison [72]. A second explanation is that post-veraison cluster exposure may accelerate carotenoid degradation, possibly by increasing *VvCCD1* expression [73], although the effect of sun exposure on increasing carotenoid degradation rates has also been disputed [55]. A third potential explanation is that sun exposure results in

conversion of epoxyxanthophylls (e.g. violaxanthin) to de-epoxidized xanthophylls (e.g. zeaxanthin). Since the putative starting point for the precursors of TDN, vitispirane, and related compounds may be de-epoxidized xanthophylls [60], sun exposure may alter the proportion of de-epoxidized vs. epoxidized forms of xanthophylls, and these different substrates could yield different C_{13}-norisoprenoid precursors post-veraison [61]. Berries exposed to sun pre-veraison are reported to have a higher proportion of de-epoxidized xanthophylls [71] than shaded berries, but a clear correlation between a specific carotenoid or carotenoids in pre-veraison grapes and eventual concentrations of TDN or other C_{13}-norisoprenoids in mature fruit has not been conclusively demonstrated.

In summary, increased cluster exposure may increase concentrations of TDN precursors and related compounds through one or more mechanisms, including greater accumulation of carotenoids, faster carotenoid degradation, or increased availability of specific carotenoid substrates. This lack of understanding of the relationship between C_{13}-norisoprenoids and light is inadequate for designing viticultural management strategies to avoid TDN precursor production while ensuring an open canopy to reduce disease and improve fruit composition. Although many reports have studied the relation of TDN precursor concentrations to cluster light exposure, none have considered altering the timing of the cluster exposure treatment. Our current study aimed to elucidate these relationships by observing the effects of cluster exposure timing on carotenoid profiles and eventual C_{13}-norisoprenoid concentrations.

Materials and Methods

Chemicals. Astaxanthin was obtained from ChromaDex (Irvine, CA). Zeaxanthin, α-carotene, β-carotene, β-damascenone, and 2-octanol were obtained from Sigma-Aldrich (St. Louis, MO). Lutein was provided as a gift from the Institute for Genomic Diversity, Cornell University. All carotenoids were ≥95% purity, and the other standards were >97% purity. NaCl and butylhydroxytoluene (BHT) were reagent grade (Fisher-Scientific, Pittsburgh, PA). Methanol, ethanol, dichloromethane, tetrahydrofuran, and petroleum were HPLC grade (Fisher-Scientific). TDN was synthesized from α-ionone (Sigma-Aldrich, 99%) via

ionene using the protocol of Miginiac [103], and the purity of the TDN standard was estimated to be >99% by NMR.

Vineyard. The field experiment was conducted during the 2008 growing season with established Riesling vines (clone 90) within a commercial vineyard. Vines had been planted on 3309 rootstock and were located on the West side of Seneca Lake in the Finger Lakes region of New York State (lat. 42.54° N, long 76.87° W). Vines were trained to a cane-pruned Scott-Henry system with 2-meter spacing between vines and 3-meter spacing between rows, with rows oriented North-South on a western facing slope. Other than the treatments described below, the test panels were managed by the commercial cooperator in the same manner as the rest of the vineyard, according to typical practices for the region.

An experimental unit consisted of an interior vineyard panel of four contiguous vines between trellis posts. A randomized complete block design was employed with four panel replicates per treatment. Test panels were inspected prior to bud-break and chosen for consistency. One 2-days PBS (past berry-set) experimental unit was removed from the study early in the season after exhibiting chlorosis and a loss of vigor. This removal resulted in each treatment consisting of four panels with a total of 16 vines except for the 2-days PBS which consisted of 3 panels with a total of 12 vines for a total of 60 vines in the study. To achieve consistency among vines in the study, shoots were thinned at 20-days before berry set to 17.5 shoots/meter, which was the lowest density found before thinning, when shoot density had varied from 17.5- 21.5 shoots/meter.

Treatments and Canopy Assessment. Three leaf removal treatments (75% of leaves in the fruiting zone removed by hand) were applied at 2-days (June 24), 33-days (July 25), and 68-days PBS (August 30), with a control where no leaf removal was administered. Berry-set was defined as when swelling had initiated and flower senescence (nearly 100%) was obvious from visual inspection. The 68-days PBS treatment was applied at approximately 3-days post-veraison, with veraison defined as the point when at least 50% of the berries had softened. Leaf removal was conducted by hand in a manner similar to

common vineyard production practices. Following leaf removal treatment, vines were allowed to refoliate, i.e. vines were not maintained at 75% leaf removal following treatment. The day following leaf removal, the canopy density was quantified for all panels using Enhanced Point Quadrat Analysis (EPQA) and analyzed using EPQA-CEM Toolkit version 1.6 [104]. EPQA was also administered at approximately 30 day intervals following leaf removal, which coincided with the day following the next leaf removal treatment, for a total of 3 EPQA sampling points. EPQA was utilized to describe canopy architecture and quantify cluster exposure, with canopy data were collected at 20cm intervals. No attempt was made in this study to separate the effects of temperature and cluster exposure, so it should be assumed that increased exposure coincided with increased cluster temperature [10, 105]. The calculated EPQA metric cluster exposure layer (CEL) was used. A lower value of CEL indicates greater cluster light exposure.

Sampling and Harvest. Carotenoid analysis was conducted on whole berry samples taken at two different periods, 52-days after berry set (mid-season) and at harvest. The mid-season sampling was prior to the final 68-days PBS treatment. Samples for C_{13}-norisoprenoid precursors were taken at harvest, with juice used for analysis. Berries were sampled from all experimental units for carotenoid analysis at 56-days after berry-set (August 17) which was 23-days after the 33-days PBS treatment, and at harvest (October 8), with 100 randomly collected berries collected from each experimental unit in duplicate. The first carotenoid samples were taken before the final leaf removal treatment. The harvest date of October 8 was dictated by the vineyard manager. Each vine was hand-harvested separately, with the number of clusters per vine and yield per vine determined. Yield and pruning weight (see below) were measured on a per vine basis using a hanging scale. Samples for analysis were frozen and held at -40^{o}C until pressing, while the rest of the harvested fruit was pressed, vinified and bottled.

Duplicate 200 ml juice samples from each experimental unit were frozen at -40^{0}C until C_{13}-norisoprenoid analysis was conducted. Pruning was conducted on February 11, 2009, leaving four canes of approximately 15 nodes each (approximately 60 buds per vine). Weight of removed prunings was

recorded on a per vine basis. Crop load was calculated on a per vine basis by calculating yield/pruning weight.

Winemaking. Fruit from all experimental units from a treatment were combined and pressed using a hydraulic basket press on the day of harvest. Wines were vinified with two replicates for each vineyard treatment, for a total of 8 fermentations. The collected juice was treated with 50 mg/L SO_2 and allowed to settle for 12 hours. Juice was racked into 19 L carboys. Juice was inoculated with *Saccharomyces cerevisiae* strain R-HST yeast (Lallemand, Inc. Toulouse, France) previously rehydrated in GoFerm (Lallemand) according to manufacturer's instructions. FermAid K (Lallemand) was added (0.13g/L) at inoculation and when wines had reached approximately 10° Brix. Wines were fermented to dryness as determined by Clinitest (Bayer, West Haven, CT), racked, cold stabilized and bottled about four months after the grapes had been pressed. No pH or sugar adjustments were performed during vinification and wines did not undergo malolactic fermentation. C_{13}-norisoprenoid analysis was conducted on wines 6 months after the grapes had been pressed and two months after bottling.

Juice Soluble Solids. The juice soluble solids content was analyzed from pressed, previously frozen samples. Samples for each experimental unit were analyzed separately, with duplicate analytical replicates. Soluble solids were measured using a Leica temperature compensating Brix scale (0-30) refractometer (Leica Inc, Buffalo, NY).

Carotenoid Analysis of Grapes. The carotenoid extraction method was adapted from a previously published method [106]. Briefly, 100 frozen berries (~60 g) were homogenized with a Waring blender divided into 25g aliquots, and astaxanthin added as an internal standard (final concentration = 100μg/kg). Carotenoids were extracted with 25 mL of 50:50 methanol / tetrahydrofuran with 0.1% BHT. Extracts were centrifuged, and the precipitate was re-extracted with methanol / tetrahydrofuran. The two

supernatant fractions were pooled and combined in a separatory funnel with 50 mL petroleum ether + 0.2% BHT (w/v) and 25 mL of aqueous NaCl (20% w/v). The organic phase was dried under a vacuum to approximately 0.5 mL, with drying finished under nitrogen, and re-dissolved in 2 mL ethanol. The extraction protocol was performed in duplicate. Carotenoid extracts were not saponified prior to analysis since during methods development zeaxanthin and the internal standard astaxanthin were not detected following saponification (data not shown). The basic conditions of saponification have been previously reported to result in oxidation of astaxanthin [107]. Additionally, stereomutation of the all-*trans* native forms of lutein and zeaxanthin to *cis* forms is accelerated at higher temperatures resulting in chromatographic peak broadening, and making it impossible to discern zeaxanthin from the lutein co-elution [107].

HPLC analysis of carotenoids was conducted using an Agilent (Santa Clara, CA) Zorbax XDB-C18 column (150 mm × 4.6 mm, 5 μm) fitted with a Zorbax XDB-C18 guard column (20 mm × 4 mm, 5 μm) on an Hewlett-Packard 1100 series HPLC system equipped with a UV/VIS diode array detector , set to record λ: 350- 600nm. The absorbance at 450 nm was used for quantification of grape carotenoids [62]. Two different HPLC gradients were employed to achieve baseline resolution of all carotenoids of interest. β-carotene, neochrome, neoxanthin and violaxanthin were analyzed by an acetone:water solvent system **(Gradient I)**: 0 to 20 min 70:30 (v/v) to 100% acetone; 20 to 30 min constant 100% acetone. The flow rate was 1 mL/min [62]. β-carotene was identified and quantified with respect to an authentic standard. Neochrome, neoxanthin and violaxanthin were identified by comparison of spectra and retention times to previous reports using the same solvent system [62], and reported as lutein equivalents.

The zeaxanthin and lutein peaks were not adequately separated by the first gradient, so an alternative gradient (**Gradient II)** was developed with the same acetone:water solvent system: 0 to 5 min 50:30 (v/v); 5 to 10 min 70:30 to 76.5:23.5, and held until 16 min; then 76.5:23.5 to 78:22 from 16 to 18 min; 78:22 to 100% acetone from 18 to 24 min; then held at 100% acetone from 24 to 35 min. The flow rate was 1 mL/min. An average resolution of 1.5 was obtained between lutein and zeaxanthin in samples. Zeaxanthin and lutein were identified and quantified with respect to authentic standards.

Analysis of Free and Total C_{13}-Norisoprenoids. For measurements of free C_{13}-norisoprenoids in wines and juices, a solid-phase extraction (SPE) protocol was adopted from conditions used in previous studies [108]. Wine and juice samples were centrifuged and filtered through #1 Whatman filter paper. The internal standard (2-octanol) was added to 50 ml of sample to yield a final concentration of 50 µg/L [108]. Samples were loaded on to SPE cartridges (Merck, Darmstadt, Germany) containing 200 mg LiChrolut EN sorbent pre-conditioned with 5 ml dichloromethane, 5 ml methanol and 10 ml H_2O. Solvent elution was facilitated by use of a Varian (Walnut Creek, CA) Cerex SPE processor and N_2 headpressure (10 psi). Following sample loading, cartridges were rinsed with 4 mL H_2O prior to elution of the analytes with 2 ml dichloromethane, and the eluent was dried under N_2 gas to a final volume of 100 µL.

Measurements of total C_{13}-norisoprenoids utilized an acid hydrolysis step prior to SPE [53, 63]. Following filtration and addition of the internal standard, samples were acidified to pH = 2.0 with 2M HCl and heated (100°C, 60 min). Due to the formation of a haze after cooling the samples, juice samples were re-filtered prior to the subsequent SPE analyses.

GC-MS analysis was conducted on a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2000 Ion Trap – MS (Walnut Creek, CA). Separation was performed on a Varian CP-Wax 58 column (40 m x 0.25 mm x 0.5 µm). The initial oven temperature was 40°C and held for 6 minutes; then ramped to 140°C @ 10°C/min.; then to 170 °C @ 5 °C/min; then to 250 °C at 10 °C/min and held at 250 °C for 20 min. The GC was operated at a constant flow rate of 1 mL/min. Three µL of extract were injected splitless, with a purge time of 0.75 min. The temperatures for the transfer line, manifold, and ion trap were 250ºC, 50ºC, and 170ºC respectively. The ion trap MS was operated over the range, *m/z* = 25-220. Data processing and quantification was performed using the native Varian Saturn GC-MS software (version 5.52). Calibration curves for β-damascenone and TDN were generated in model juice and wine against the 2-octanol internal standard over a range of 1-300 µg/L for TDN and 0.1-30 µg/L for β-damascenone. Since standards were not available, vitispirane A and B were identified by retention index and MS library spectra (NIST Mass Spectral Library version 1.7a) and the sum of the isomers reported as

TDN equivalents. The following ions were used for quantification and identification: β-damascenone (quantification ion *m/z*=121, qualifier ion *m/z*=69 and 175), TDN (157, 172 and 142), vitispirane A (192, 177 and 93), vitispirane B (177, 192 and 121). Peak definition and quantification was based on the selected ion chromatograms from the full mass spectral data set.

Statistical analysis. Statistical analysis was conducted using SAS JMP version 8.0 (SAS Cary, NC) for standard deviation, and Tukey-Kramer HSD, and linear regression. Welch's t-test [109] was conducted in Microsoft Excel 2007 (Redmond, WA). A maximum p-value less than 0.05 was necessary for results to be reported as significant.

Results And Discussion

Impact of Leaf Removal Treatments on Canopy Microclimate and Fruit Maturity. EPQA was performed on the canopies of all experimental units, one day after each leaf removal treatment was applied, to assess the effects of the treatments on canopy architecture and cluster light environment (Figure 5.2) This also allowed assessment of canopy re-growth in those treatments that had undergone leaf removal earlier in the season. CEL measures the average number of occlusions experienced by clusters, and thus is an indicator of cluster light exposure, where lower CEL indicates greater cluster exposure. Berry temperature was not measured, but previous reports have observed an increase in berry temperature with increased cluster light exposure [10, 110] and it is difficult to decouple these parameters experimentally [105]. As expected, significantly lower CEL was observed for 33-days and 68-days PBS treatments immediately following their respective leaf removal events compared to the other

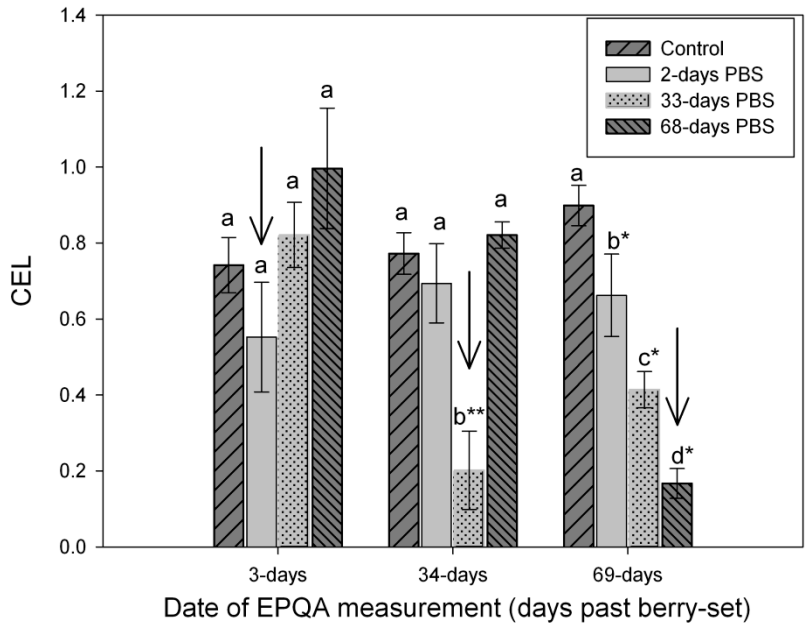

Figure 5.2. Effect of leaf removal treatment on cluster exposure layer (CEL) measured one day after each treatment application.

Measurements were taken 3-days (June 25), 34-days (July 26) and 69-days (August 31) after berry set, corresponding to the days following each leaf removal treatment. Veraison was approximately 65-days after berry set. The control received no leaf removal. Different lower-case letters at same date of measurement (a, b, c, d) indicate difference in means by Tukey HSD at a significance level of *= $p < 0.05$, **= $p < 0.01$. Error bars indicate one standard deviation. An arrow indicates the treatment that received leaf removal the previous day.

treatments ($p<0.01$). The 2-days PBS treatment did not result in significantly lower CEL than the control (0.55 vs. 0.72) when quantified at 3-days after berry set, however we did observe a CEL significantly lower than the control for the 2-days PBS treatment following the 68-days PBS leaf removal (0.66 vs.0.90 , $p<0.01$). The treatments had little effect on yield and yield components (Table 5.3). There were no significant differences among treatments for yield per vine or average cluster weight, however the pruning weight for the 2-days PBS treatment (1.59 kg/vine) was higher than the control (1.31 kg/vine), and 33-days PBS (1.28 kg/vine, , $p<0.05$). These results suggest the 2-days PBS treatment may have induced vegetative growth outside of the fruiting zone, since the CEL was significantly lower than the control at the final EPQA (69-days after berry-set). Cropload (vine yield / pruning weight) was significantly lower for the 2-days PBS treatment than the 33-days PBS treatment (9.05 vs. 7.07, $p<0.05$), due to the higher pruning weight values for the 2-days PBS treatment. Soluble solids of the 2-days PBS treatment were significantly higher than that of 68-days PBS (20.3° vs. 18.7° Brix), but no difference was

observed with respect to the treatments compared to the control. Early leaf removal has been previously reported to increase Brix and advance fruit maturity [58, 59].

Table 5.1. Mean vine growth and crop measurements for leaf removal treatments.

	Treatment (Leaf removal timing)[a]							
	Control[b]	SD	2-days PBS	SD	33-days PBS	SD	68-days PBS	SD
cluster count/vine	92.3 a	4.2	90.6 a	6.7	98.6 a	3.9	88.0 a	4.5
fruit weight (kg/vine)	11.0 a	0.6	11.0 a	0.4	11.4 a	0.9	11.6 a	0.5
mean cluster weight (kg)	0.12 a	0.00	0.12 a	0.00	0.12 a	0.01	0.13 a	0.00
pruning weight (kg/vine)	1.3a	0.1	1.6b*	0.1	1.3a	0.0	1.4ab	0.1
crop load Index [c]	8.7[ab]	0.9	7.1[a]	0.4	9.1[b*]	0.7	8.8[ab]	0.2
total soluble solids	19.1[ab]	0.8	20.3[a]	0.6	19.1[ab]	0.2	18.7[b*]	1.1

[a] Treatment timings refer to the days past berry-set (PBS) for fruit zone leaf removal, with the control receiving no leaf removal. [b]Using Tukey HSD significance of means among all experimental units were compared. Different lower-case letters within a row (a, b), with significance level of *= $p < 0.05$ [c] Crop load index was calculated by fruit weight/ pruning weight.

Berry Carotenoids. Quantification of four carotenoids (β-carotene, neochrome, neoxanthin and violaxanthin) was performed by a previously described C_{18}-HPLC protocol (Gradient I, Figure 5.3) [73, 111]. Using HPLC Gradient I, quantification of zeaxanthin was not possible due to its co-elution with lutein. Previous reports investigating the impact of sun exposure on berry carotenoids have

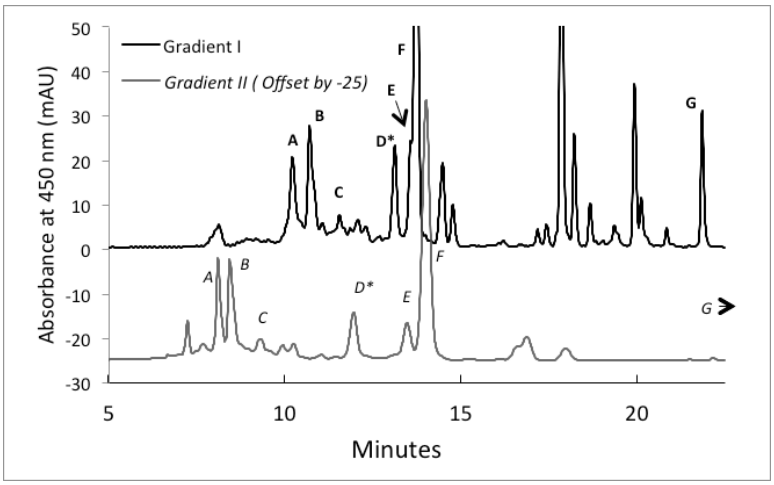

Figure 5.3. HPLC chromatogram (450 nm) of carotenoids in a harvest sample using Gradient I and Gradient II (signal offset by -25 on plot).

(A) neoxanthin (B) neochrome (C) violaxanthin (D*) astaxanthin, internal standard (E) zeaxanthin (F) lutein (G) β-carotene. Gradient I labeled in bold, Gradient II in italics.

observed a similar co-elution using this protocol, which accounts for the limited reporting of zeaxanthin in gape related literature [61, 71, 73, 111]. Quantification of the total de-epoxidized xanthophyll pool in grapes, including zeaxanthin, has been previously reported using a non-endcapped reverse phase HPLC column [71]. However, non-endcapped columns are not widely used due to increased peak tailing from free silanol groups. Using a conventional endcapped column, we modified the solvent gradient (Gradient II) to yield acceptable baseline resolution between lutein and zeaxanthin (R_s = 1.5) (Peaks E and F, respectively, Figure 5.3). Using Gradient II, we also observed that the β-carotene peak obscured a small α-carotene peak (<10% β-carotene peak area), confirmed by comparison to an authentic standard (data not shown). The separation was not adequate for quantification of α-carotene. To our knowledge, α-carotene has not been previously reported in grapes.

Total berry carotenoid concentrations decreased during maturation from 1,500-2,700 µg/kg at mid-season to 330-880 µg/kg at harvest (Table 5.2), similar to previous reports [10, 60]. The major carotenoid species

Table 5.2. Berry carotenoid concentration (mean and standard deviation, SD) for mid-season (52-days after berry set) and harvest (107-days after berry set) samples.

			Treatment (leaf removal timing)							
			Control		2-days PBS		33 -PBS		68-days PBS	
Carotenoid			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lutein	Mid-season	µg/kg	971^{a}	145	737^{a}	86	902^{a}	58	939^{a}	46
		% of Total	50^{a}	3	46^{a}	3	47^{a}	1	46^{a}	2
	Harvest	µg/kg	280^{a}	60	301^{a}	27	300^{a}	43	189^{a}	32
		% of Total	53^{ab}	3	59^{a}	3	47^{ab}	1	43^{b*}	5
β-carotene	Mid-season	µg/kg	405^{a}	137	348^{a}	153	391^{a}	90	497^{a}	62
		% of Total	21^{a}	5	19^{a}	6	19^{a}	4	24^{a}	2
	Harvest	µg/kg	45^{a}	14	49^{a}	28	84^{a}	27	27^{a}	8
		% of Total	9^{a}	2	9^{a}	4	12^{a}	3	6^{a}	1
Zeaxanthin	Mid-season	µg/kg	88^{a}	13	63^{a}	2	142^{b*}	14	93^{a}	4
		% of Total	5^{a}	0	4^{a}	1	7^{b***}	1	5^{a}	0
	Harvest	µg/kg	24^{a}	2	39^{a}	3	39^{a}	2	36^{a}	6
		% of Total	5^{a}	1	8^{ab}	1	6^{ab}	1	8^{b*}	1
Neoxanthin	Mid-season	µg/kg	159^{a}	24	175^{a}	20	164^{a}	17	158^{a}	9
		% of Total	8^{ab}	0	11^{a}	1	9^{ab}	1	8^{b*}	0
	Harvest	µg/kg	58^{a}	12	44^{a}	3	77^{a}	11	65^{a}	10
		% of Total	11^{ab}	2	9^{a}	1	12^{ab}	1	15^{b*}	2
Neochrome	Mid-season	µg/kg	232^{a}	25	243^{a}	20	254^{a}	19	261^{a}	33
		% of Total	12^{a}	1	15^{a}	1	13^{a}	1.9	13^{a}	1
	Harvest	µg/kg	87^{a}	24	52^{a}	13	85^{a}	3	77^{a}	16
		% of Total	16^{ab}	2	10^{a}	1	14^{ab}	2	18^{b*}	4
Violaxanthin	Mid-season	µg/kg	75^{a}	10	76^{a}	11	87^{a}	6	85^{a}	4
		% of Total	4^{a}	0	5^{a}	0	5^{a}	0	4^{a}	0
	Harvest	µg/kg	39^{a}	9	27^{a}	1	47^{a}	8	41^{a}	5
		% of Total	7^{ab}	1	5^{a}	1	7^{ab}	0	9^{b*}	1

Treatment timings refer to the days past berry-set (PBS) for fruit zone leaf removal, with the control receiving no leaf removal. At the mid-season time point, leaf removal had not been performed on the 68-days PBS treatment, and was thus similar to the control. Differences among treatments were determined by Tukey HSD. Different lower-case letters within a row (a, b), with significance level of *= $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.005$.

were β-carotene and lutein, and these two compounds summed to account for 69% (mid-season) and 60% (harvest) of the total measured carotenoids. This is lower proportionally than values around 85% reported previously for other varieties of grapes [73]. We observed no significant differences among treatments for total carotenoids at either sampling time point. The existing literature on the impact of pre-veraison cluster light environment on carotenoids is inconsistent [70, 72]. Pre-veraison cluster shading has been reported to lead to lower carotenoid content mid-season [70], as would be expected from the general observation that sunlight can stimulate Photosystem II activity in plants, and consequentially carotenoid biosynthesis [112]. However, at least one study has observed higher concentrations of total carotenoids in shaded grapes during development [113], which has been observed in other higher plants, especially those grown in extreme shade [114]. It was suggested in these studies that the increased carotenoids in shade-grown plants are used as light harvesting antennae, or for protection from brief periods of direct sun. Similarly, some previous studies have observed higher total carotenoid concentrations in mature shaded fruit [69, 73], putatively because light exposure accelerates photo- or enzymatic degradation *in vivo*, but this result was not observed in another study [72], and no change in carotenoid degradation rate was observed in vines exposed to partial root zone drying, despite increased cluster sun-exposure [55].

Of the six individual carotenoids quantified in our study, only zeaxanthin showed a significant response to any treatment when considering absolute concentrations, though treatment effects were observed on other carotenoids when expressed as a percentage of the total carotenoid pool (Table 5.2). In the 33-days PBS treatment samples, zeaxanthin at mid-season had a mean concentration of 142 μg/kg, significantly greater ($p<0.05$) than the control, 2-days PBS and 68-days PBS treatment samples (range = 63-93 μg/kg). In plants, zeaxanthin is formed either from β-carotene or via de-epoxidation of epoxyxanthophylls (e.g. violaxanthin) as part of the xanthophyll cycle to dissipate excess energy during photosynthesis [68, 115]. The mid-season samples also showed a significant increase ($p<0.005$) in the proportion of zeaxanthin relative to total carotenoids, with zeaxanthin constituting 7% of total carotenoids in 33-days PBS treatment versus 4-5% for other treatments. A significant correlation of zeaxanthin as a percentage of total carotenoids (% zeaxanthin) at mid-season (56-days after berry set) vs. CEL taken 34-

days after berry set was found (R^2=0.83, p<0.0001, data not shown). This correlation was particularly strong when considering only the four experimental units of the 33-days PBS treatment (R^2= 0.99 p=0.005, plot not shown), but significant correlations were not observed within the other treatments, possibly because of a narrower range of zeaxanthin concentrations (Table 5.2). The increase in zeaxanthin in mid-season berries in the 33-days PBS treatment is expected, since sun exposure and resulting PSII overexcitation is reported to increase the total xanthophyll pool [71] as well as the ratio of de-epoxidized xanthophylls to epoxidized xanthophyll forms [115]. At harvest, no difference in the absolute zeaxanthin concentration was observed between the 33-days PBS treatment and the control. This may indicate greater enzymatic degradation of zeaxanthin via *VvCCD* in the 33-days PBS treatment after veraison, although it is also possible that zeaxanthin is recycled to an epoxy form prior to carotenoid degradation.

No difference was observed among the 68-days PBS treatment and other treatments at either time point,. However, a significantly higher zeaxanthin proportion was observed at harvest in the 68-days PBS treatment compared to the control (8% vs. 5%). As mentioned previously, sun exposure is reported to increase the proportion of zeaxanthin in the carotenoid pool. The lack of a significant impact of the 68-days PBS treatment on absolute zeaxanthin concentrations at harvest as compared to the 33-days PBS treatment at mid-season may have been due to the larger gap between the treatment timing and the carotenoid sampling point, and the resultant change in light environments likely caused by canopy growth; 39-days elapsed between the 68-days PBS treatment and the harvest date, as compared to 22-days difference between the 33-days PBS treatment and the mid-season sampling time point. In comparison to the % zeaxanthin values, absolute zeaxanthin concentration will be more influenced by other factors regulating total carotenoid concentration, e.g. berry size, and as a result suffer from more biological variability. Additionally, carotenoid production in post-veraison grapes has been demonstrated to be minimal [61].

Sun exposure has been reported to deplete epoxyxanthophylls in some plants [116] but no significant difference was observed for neoxanthin among treatments in our work with respect to the

control at either time point. Interestingly, the 2-days PBS treatment had a higher proportion of lutein and a lower proportion of neoxanthin in mid-season samples than the 68-days PBS treatment, although no difference was observed compared to the control. The 2-days PBS treatment also had a lower violaxanthin and neochrome proportion in mature fruit samples. The reason for these differences is not apparent but may be related to the increased fruit maturity observed in the 2-days PBS over the 68-days PBS treatment as measured by soluble solids.

Free and Total C_{13}-Norisoprenoids in Juice. Concentrations of free and total (free + bound) C_{13}-norisoprenoids were quantified in both juice and wine. Although glycosylated precursors can be liberated either enzymatically or by acid-hydrolysis during winemaking, we selected acid hydrolysis for determining total C_{13}-norisoprenoids because the species of interest (TDN, β-damascenone, vitispirane) are not observed under enzymatic hydrolysis conditions [61]. Acid-hydrolysis under heated conditions has the additional benefit of evolving potential non-glycosylated precursors [63]. Additionally, TDN and vitispirane concentrations are observed to increase dramatically during storage [53], so acid-hydrolysis would be expected to better reflect the total potential concentrations of these compounds in particular.

Mean concentrations of free and total C_{13}-norisoprenoids in juice and wine are shown in Table 5.3. In the juice samples, free TDN and vitispirane were below the method's detection threshold (<0.1 µg/L as TDN equivalents), and only trace levels (below quantification limit, <0.3 µg/L) of free β-damascenone were detectable in the juice samples. This is in concordance with previous reports which have observed undetectable or trace levels of C_{13}-norisoprenoids in juice [10].

Significantly higher concentrations of total, acid-liberated TDN and vitispirane were observed in the 33-days PBS treatment juice samples compared to the control and the other treatments ($p < 0.05$). The mean total TDN concentration was 195 ug/L for the 33-days PBS treatment, vs. 54-81 ug/L for the other treatments (Table 5.3). Cluster light exposure has been previously linked to increased concentrations of TDN/vitispirane precursors in harvested fruit [52], but the critical time period during

Table 5.3. C_{13}-norisoprenoid concentrations for juice and wine samples from each leaf removal treatment.

			Treatment (leaf removal timing)							
			Control		2-days PBS		33-days PBS		68-days PBS	
Norisoprenoid (µg/L)			Mean	SD	Mean	SD	Mean	SD	Mean	SD
TDN	Juice	Free	ND		ND		ND		ND	
		Total	71[a]	12	54[a]	9	195[b**]	26	87[a]	22
	Wine	Free	9[a]	0	7[a]	1	20[b*]	1	11[a]	2
		Total	52[a]	16	61[ab]	2	138[b*]	31	81[ab]	12
Vitispirane A+B (as TDN equivalents)	Juice	Free	ND		ND		ND		ND	
		Total	37[a]	5	30[a]	3	56[b*]	10	32[a]	5
	Wine	Free	10[a]	2	7[a]	1	9[a]	0	9[a]	1
		Total	122[a]	4	109[a]	11	132[a]	8	129[a]	13
β-damascenone	Juice	Free	NQ		NQ		NQ		NQ	
		Total	10[a]	1	7[ab]	1	10[a]	1	7[b*]	1
	Wine	Free	6[a]	1	8[a]	1	5[a]	0	9[a]	0
		Total	4[a]	0	9[a]	1	4[a]	2	8[a]	1

ND = not detected (< 1 µg/L for TDN, 1< µg/L for vitispirane).

NQ = not quantifiable (0.1 < damascenone < 0.3 µg/L).

Total refers to the concentration following acid hydrolysis. Standard deviations (SD) were calculated from treatment replicates for juices (n= 3 or 4). The juices were pooled prior to winemaking, and mean and SD were calculated for the winemaking replicates (n=2). Treatment timings refer to the days past berry-set (PBS) for fruit zone leaf removal, with the control receiving no leaf removal. Different lower-case letters within a row (a, b) indicate difference in means by Tukey HSD at a significance level of *= $p < 0.05$, ** = $p < 0.01$.

timing increased acid-hydrolysable TDN/vitispirane precursors in juice. However, neither 2-days PBS nor 68-days PBS treatments increased TDN/vitispirane precursors in juice suggesting that the critical time during the growing season for forming TDN/vitispirane precursors is ~33-days after berry set. The lack of which exposure impacts precursor formation has not been established. In our current work, the 33-days PBS treatment a significant effect by the 68-days PBS treatment also indicates that cluster light exposure

does not induce formation of TDN/vitispirane precursors by increasing the rate of carotenoid degradation and C_{13}-norisoprenoid formation post-veraison.

β-damascenone concentrations in juice samples were lower in the 68-days PBS treatment than the control and 33-days PBS treatment (Table 5.3). No differences were observed among the 2-days and 33-days PBS treatments and the control ($p < 0.05$). Unlike TDN, total β-damascenone concentrations in wines have been reported to decrease in response to cluster exposure [10, 52].

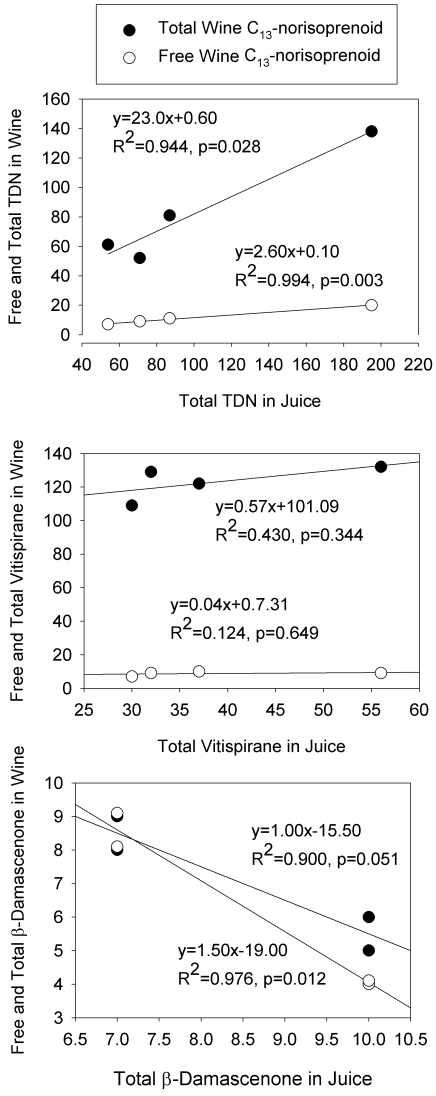

Figure 5.4. Comparison of the relationship between the means of total, acid-hydrolyzable C_{13}-norisoprenoids in juice to the free and total concentrations found in wine for TDN, vitisipirane and β-damascenone. All values are given in μg/L.

Free and Total C_{13}-Norisoprenoids in Wine and Correlation with Juice. In finished wines, total TDN was significantly higher in the 33-days PBS compared to the control and free levels were higher than the other treatments and control (Table 5.3). No differences in the concentrations of vitispirane or β-damascenone were observed between treatments in wine (Table 5.3). Free TDN in wine was >2-fold higher in the 33-days PBS treatment than the control. The 20 µg/L concentration of TDN in the 33-days PBS treatment is equal to the reported sensory threshold in wine [8]. Mean concentrations of total TDN in grapes were significantly correlated with free TDN and total TDN concentrations in wine (Figure 5.4). The mean conversion rate of total grape TDN to free TDN in wine was 11% ± 2.5%. Across treatments, we observed no significant change in total TDN in juice vs. wine, i.e. the fermentation did not result in a significant change in the total, i.e. free + potential, TDN pool (Figure 5.5). These findings suggest that total TDN in juice is a good indicator of total TDN in wine.

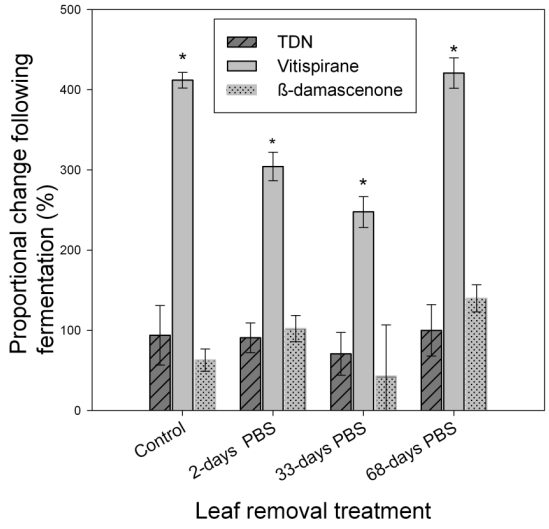

Figure 5.5. Change in total (free + potential) C_{13}-norisoprenoids resulting from winemaking, calculated as the ratio of C_{13}-norisoprenoids to C_{13}-norisoprenoids in must x 100%.

By definition, 100% indicates identical concentrations in juice and wine. Error bars indicate one standard deviation. * indicates that C_{13}-norisoprenoid concentration in wine was significantly different than C_{13}-norisoprenoid concentration in must ($p < 0.0001$, Welch's t-test, n=3 or 4 for juice, n=2 for wine).

The β-damascenone pool in wine existed entirely in the free form, as no increase was observed in β-damascenone following acid hydrolysis (Table 5.3). The mean conversion rate of total grape β-

damascenone to free β-damascenone in wine was quantitative, 81% ± 35% (Figure 5.5). In contrast, the majority of TDN still existed in the form of bound precursors following fermentation. The faster kinetics of β-damascenone formation may be due to different rates of acid or enzymatic hydrolysis or rearrangement on precursors during winemaking. Interestingly, we observed a significant negative correlation of total β-damascenone in juice with free β-damascenone in wine (Figure 5.4). The reason for this phenomenon is unclear, but a potential explanation is that the fruit with low total β-damascenone may have had other precursors that were transformed to β-damascenone precursors during fermentation.

Free and total concentrations of vitispirane in wine were not correlated with total vitispirane in juice (Figure 5.4). We observed a significant increase (mean = 91 ± 10 μg/kg) in total vitispirane following fermentation compared with juice samples (Figure 5.5), or a 330% increase in total vitispirane (Figure 5.5). To our knowledge, an increase in total vitispirane in wine with respect to grape juice has not been previously reported, since direct or indirect measurement of potential volatiles in wine is rarely reported. An increase in total vitispirane was observed previously when apple leaf glycoside extract was fermented with baker's yeast [117]. The authors proposed this resulted from enzymatic reduction of glycosylated TDN precursors to glycosylated vitispirane precursors. While this could explain the increase of total vitispirane in our study, it does not explain why total TDN did not show a corresponding decrease. Regardless, these results show that predicting vitispirane concentration in finished wines based on acid hydrolysis is not advisable.

Relation of C_{13}-Norisoprenoids to Carotenoids and Cluster Exposure. Our current work indicates that pre-veraison cluster exposure (33-days PBS) by leaf removal will significantly increase TDN and vitispirane precursors in juice compared to exposure at berry set or post-veraison. The pre-veraison leaf removal timing also results in significantly higher free and total TDN in wine. Though total carotenoid content was not affected by any treatment, an increase in zeaxanthin in the 33-days PBS treatment was observed in mid-season berry samples.

The observation that both zeaxanthin and total TDN increase in the 33-days PBS treatment is intriguing as zeaxanthin has been demonstrated to generate two putative precursors of TDN *in vitro* via photo-oxidation to yield 3-hydroxy-5,6-epoxy –β-ionone [118], or enzymatic degradation to yield 3-hydroxy-β-ionone [50]. Thus, it is possible that zeaxanthin may be a precursor for grape-derived glycosylated TDN precursors *in vivo* as well. We observe a significant correlation between mid-season zeaxanthin and total juice TDN (R^2=0.59, p=0.0009, plot not shown) and % zeaxanthin and total juice TDN (R^2=0.68, p=0.0003, plot not shown). However, within treatments, a significant correlation was only observed for the four 33-days PBS treatment replicates (R^2=0.96, p=0.02, plot not shown). This may be because of the narrower range of TDN in the treatments other than 33-days PBS and the greater proportional importance of noise, or that the zeaxanthin – TDN relationship is correlative rather than causal. The peak concentration of zeaxanthin in grapes is unknown, so it is also possible that we did not capture the maximum zeaxanthin concentration. Since other carotenoids have also been reported to yield TDN *in vitro*, e.g. lutein following acid hydrolysis [50, 63], cell culture or labeling studies with putative precursors may be necessary to distinguish the critical pathways *in vivo*.

Implications with Respect to Cultural Practices: Our findings could have important implications for selecting cultural practices to target specific flavor profiles. Excessive concentrations of TDN in young wines are sometimes reported to be undesirable [69], likely because strong "petrol" aromas would mask other Riesling aroma attributes. Reducing cluster light exposure during the growing season is one strategy for growers interested in reducing the eventual concentration of TDN in wines. However, as mentioned in the introduction, increasing berry sun exposure is often desirable for reducing disease pressure, decreasing titratable acidity, and effecting other desirable changes to fruit qualities. The results of our study indicate that the key period during the growing season associated with production of acid-releasable TDN precursors is pre-veraison (33-days PBS treatment). Growers could implement leaf removal at berry set or post-veraison for disease control, etc., without a resulting increase in TDN.

Conversely, pre-veraison leaf removal could be employed if higher TDN concentrations were desired in wine.

In summary, we have demonstrated that the timing of leaf removal can alter the mid-season carotenoid profile, as well as TDN and vitispirane precursors in mature Riesling grapes. Leaf removal at 33-days PBS resulted in elevated mid-season zeaxanthin concentrations, elevated total TDN/vitispirane in juice, and elevated free TDN in wine as compared to other treatments and the control. Therefore, our results suggest that leaf removal can be practiced at berry-set and post-veraison without a significant effect on TDN or vitispirane potential. However, the implication that zeaxanthin is the source of TDN/vitispirane precursors still needs to be evaluated in future studies. β-damascenone in wine was unaffected by the leaf removal treatments, in concordance with previous reports indicating differential regulation of β-damascenone and TDN/vitispirane precursors. Finally, total vitispirane increased by up to 4-fold after fermentation, indicating that the conditions associated with fermentation may transform glycosylated precursors, and that potential vitispirane in grapes is a poor predictor of vitispirane post-fermentation.

References

1. Rapp, A. Volatile flavour of wine: Correlation between instrumental analysis and sensory perception. *Nahrung-Food* **1998,** *42,* 351-363.

2. Mathieu, S.; Terrier, N.; Procureur, J.; Bigey, F.; Gunata, Z. A Carotenoid Cleavage Dioxygenase from Vitis vinifera L.: functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. *J. Exp. Bot.* **2005,** *56,* 2721-2731.

3. Jesús Ibarz, M.; Ferreira, V.; Hernández-Orte, P.; Loscos, N.; Cacho, J. Optimization and evaluation of a procedure for the gas chromatographic–mass spectrometric analysis of the aromas generated by fast acid hydrolysis of flavor precursors extracted from grapes. *Journal of Chromatography A* **2006,** *1116,* 217-229.

4. Simpson, R.F. 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene: An important contributor to the bottle aged bouquet of wine. *Chem. Ind.* **1978,** *1,* 37.

5. Marais, J.; Van Wyk, C.; Rapp, A. Effect of storage time, temperature and region on the levels of 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene and other volatiles, and on quality of Weisser Riesling wines. *South African Journal for Enology and Viticulture* **1992,** *13,* 33-44.

6. Winterhalter, P.; Sefton, M.A.; Williams, P.J. Volatile C-13-Norisoprenoid Compounds in Riesling Wine are Generated from Multiple Precursors. *Am. J. Enol. Vitic.* **1990,** *41,* 277-283.

7. Lee, S.; Seo, M.; Riu, M.; Cotta, J.P.; Block, D.E.; Dokoozlian, N.K.; Ebeler, S.E. Vine microclimate and norisoprenoid concentration in cabernet sauvignon grapes and wines. *Am. J. Enol. Vitic.* **2007,** *58,* 291-301.

8. Linsenmeier, A.W.; Lohnertz, O. Changes in norisoprenoid levels with long-term nitrogen fertilisation in different vintages of Vitis vinifera var. riesling wines. *South African Journal of Enology and Viticulture* **2007,** *28,* 17-24.

9. Bindon, K.A.; Dry, P.R.; Loveys, B.R. Influence of plant water status on the production of C-13-norisoprenoid precursors in Vitis vinifera L. Cv. Cabernet Sauvignon grape berries. *J. Agric. Food Chem.* **2007,** *55,* 4493-4500.

10. Marais, J. The significance of 1, 1, 6-trimethyl-1, 2-dihydronaphthalene in the production of high quality Riesling wines, In *ACS Symposium Series 802, Carotenoid-derived aroma compounds,* Winterhalter, P. and Rouseff, R.L., Eds.; Washington, DC; American Chemical Society: 2002; Vol.802 pp. 273.

11. Marais, J.; Wyk, C.J.; Rapp, A. Effect of sunlight and shade on norisoprenoid levels in maturing Weisser Riesling and Chenin blanc grapes and Weisser Riesling wines. *South African Journal for Enology and Viticulture* **1992,** *13,* 23-32.

12. Ristic, R.; Downey, M.O.; Iland, P.G.; Bindon, K.; Francis, I.L.; Herderich, M.; Robinson, S.P. Exclusion of sunlight from Shiraz grapes alters wine colour, tannin and sensory properties. *Aust J Grape Wine Res* **2007,** *13,* 53.

13. Zoecklein, B.W.; Wolf, T.K.; Duncan, N.W.; Judge, J.M.; Cook, M.K. Effects of fruit zone leaf removal on yield, fruit composition, and fruit rot incidence of Chardonnay and White Riesling (Vitis vinifera L.) grapes. *Am. J. Enol. Vitic.* **1992,** *43,* 139.

14. Reynolds, A.G.; Wardle, D.A.; Naylor, A.P. Impact of training system, vine spacing, and basal leaf removal on Riesling. Vine performance, berry composition, canopy microclimate, and vineyard labor requirements. *Am. J. Enol. Vitic.* **1996,** *47,* 63-76.

15. Mendes-Pinto, M.M. Carotenoid breakdown products the—norisoprenoids—in wine aroma. *Arch. Biochem. Biophys.* **2009,** *483,* 236-245.

16. Baumes, R.; Wirth, J.; Bureau, S.; Gunata, Y.; Razungles, A. Biogeneration of C-13-norisoprenoid compounds: experiments supportive for an apo-carotenoid pathway in grapevines. *Anal. Chim. Acta* **2002,** *458,* 3-14.

17. Razungles, A.J.; Babic, I.; Sapis, J.C.; Bayonove, C.L. Particular behavior of epoxy xanthophylls during veraison and maturation of grape. *J. Agric. Food Chem.* **1996,** *44,* 3821-3825.

18. Marais, J. 1, 1, 6-Trimethyl-1, 2-dihydronaphthalene (TDN): a possible degradation product of lutein and beta-carotene. *South African Journal of Enology and Viticulture* **1992,** *13,* 52-55.

19. Auldridge, M.E.; McCarty, D.R.; Klee, H.J. Plant carotenoid cleavage oxygenases and their apocarotenoid products. *Curr. Opin. Plant Biol.* **2006,** *9,* 315-321.

20. Mathieu, S.; Wirth, J.; Sauvage, F.X.; Lepoutre, J.P.; Baumes, R.; Gunata, Z. Biotransformation of C 13-norisoprenoids and monoterpenes by a cell suspension culture of cv. Gamay (Vitis vinifera). *Plant Cell, Tissue and Organ Culture* **2009,** *97,* 203-213.

21. Meyers J., Sacks G., and Vanden Heuvel J. Naturally occurring spatial variability in canopy biomass impacts flavor and aroma compounds in riesling. *Am. J. Enol. Vitic.* **2009,** *60,* 394a-395a.

22. Winterhalter, P.; Schreier, P. C13-Norisoprenoid glycosides in plant tissues: An overview on their occurrence, composition and role as flavour precursors. *Flavour Fragrance J.* **1994,** *9,*

23. Hirschberg, J. Carotenoid biosynthesis in flowering plants. *Curr. Opin. Plant Biol.* **2001,** *4,* 210-218.

24. Marais, J.; Van Wyk, C.J.; Rapp, A. Carotenoid levels in maturing grapes as affected by climatic regions, sunlight and shade. *S.Afr.J.Enol.Vitic* **1991,** *12,* 64-69.

25. Bureau SM; Razungles AJ; Baumes RL; Bayonove CL Effect of vine or bunch shading on the carotenoid composition in Vitis vinifera L. berries. I. Syrah grapes. *Wein-Wissenschaft Viticultural and Enological Sciences* **1999,** *53,* 53 (2) 64-71.

26. Düring, H.; Davtyan, A. Developmental changes of primary processes of photosynthesis in sun- and shade-adapted berries of two grapevine cultivars. *Vitis* **2002,** *41,* 63-67.

27. Oliveira, C.; Ferreira, A.C.; Costa, P.; Guerra, J.; de Pinho, P.G. Effect of some viticultural parameters on the grape carotenoid profile. *J. Agric. Food Chem.* **2004,** *52,* 4178-4184.

28. Razungles, A.J.; Baumes, R.L.; Dufour, C.; Sznaper, C.N.; Bayonove, C.L. Effect of sun exposure on carotenoids and C-13-norisoprenoid glycosides in Syrah berries (Vitis vinifera L.). *Sci. Aliments* **1998,** *18,* 361-373.

29. Miginiac, P. A Facile Synthesis of 1,1,6-Trimethyl-1,2-Dihydronaphthalene. *Synth. Comm.* **1990,** *20,* 1853-1856.

30. Meyers, J.M.; Vanden Heuvel, J.E. Enhancing the Precision and Spatial Acuity of Point Quadrat Analyses via Calibrated Exposure Mapping. *Am. J. Enol. Vitic.* **2008,** *59,* 425-431.

31. Spayd, S.E.; Tarara, J.M.; Mee, D.L.; Ferguson, J.C. Separation of sunlight and temperature effects on the composition of Vitis vinifera cv. Merlot berries. *Am. J. Enol. Vitic.* **2002,** *53,* 171.

32. Wrolstad, R.E.; Acree, T.E.; Decker, E.A. *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components.* Wiley-Interscience: 2005; pp. 91-96.

33. Goodwin, T.W. Chemistry and biochemistry of plant pigments, In Second ed.; Goodwin, T.W., Ed.; Academic press New York: 1976; Vol.1 pp. 200-211.

34. Lopez, R.; Aznar, M.; Cacho, J.; Ferreira, V. Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. *J. Chromatogr. A* **2002,** *966,* 167-177.

35. Ruxton, G.D. The unequal variance t-test is an underused alternative to Student's t-test and the Mann-Whitney U test. *Behav. Ecol.* **2006,** *17,* 688.

36. Mendes-Pinto, M.M.; Silva Ferreira, A.C.; Oliveira, M.B.P.P.; Guedes De Pinho, P. Evaluation of some carotenoids in grapes by reversed- and normal-phase liquid chromatography: A qualitative analysis. *J. Agric. Food Chem* **2004,** *52,* 3182-3188.

37. Albrecht, M.; Sandmann, G. Light-stimulated carotenoid biosynthesis during transformation of maize etioplasts is regulated by increased activity of isopentenyl pyrophosphate isomerase. *Plant Physiol.* **1994,** *105,* 529.

38. Oliveira, C.; Ferreira, A.C.; Costa, P.; Guerra, J.; de Pinho, P.G. Effect of some viticultural parameters on the grape carotenoid profile. *J. Agric. Food Chem.* **2004,** *52,* 4178-4184.

39. Amiard, V.; Mueh, K.E.; Demmig-Adams, B.; Ebbert, V.; Turgeon, R.; Adams, W.W. Anatomical and photosynthetic acclimation to the light environment in species with differing mechanisms of phloem loading. *Proc. Natl. Acad. Sci. U. S. A.* **2005,** *102,* 12968.

40. Demmig-Adams, B.; Adams, W.W. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends Plant Sci.* **1996,** *1,* 21-26.

41. Thayer, S.S.; Björkman, O. Leaf xanthophyll content and composition in sun and shade determined by HPLC. *Photosynthesis Res.* **1990,** *23,* 331-343.

42. Knapp, H.; Straubinger, M.; Stingl, C.; Winterhalter, P. Analysis of Norisoprenoid Aroma Precursors, In *ACS Symposium Series 802, Carotenoid Derived Aroma Compounds,* Winterhalter, P. and Rouseff, R.L., Eds.; Washington, DC; American Chemical Society;: 2002; Vol.802 pp. 20-35.

43. Isoe, S.; Hyeon, S.B.; Katsumura, S.; Sakan, T. Photo-oxygenation of carotenoids. II. The absolute configuration of loliolide and dihydroactinidiolide. *Tetrahedron Lett.* **1972,** *13,* 2517-2520.