Phenolic Composition of Wine after Fungicide Applications and Drought Stress

A Senior Project

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By

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Introduction and Literature Review

Grape vines, like all crops, experience stress throughout the growing season, ranging from pathogen attacks to water stress. In response to these factors, plants produce secondary metabolites, like phenolics. Phenolics are compounds with a phenyl, or benzene ring attached to an –OH functional group. While they aren't necessary for growth, they exist as a defense mechanism to the plant against these stressors. By the same token, these phenolics also influence wine quality, including taste, color, and mouth feel. This study will specifically look at phenolic compound levels in Cabernet Sauvignon and Chardonnay wine samples, from a field study described below.

Viticulturists constantly work to negate the effects of stresses to their vines, including making fungicide applications regularly to combat pathogen attacks. A trial was conducted in 2012 at Scheid vineyards, San Lucas, Monterey County, to test the effects of fungicide applications or deficit irrigation on vine yield and wine quality, including phenolic concentration (Hudson, 2012). The fungicide treatments were trifloxystrobin (Flint®), quinoxyfen (Quintec®), myclobutanil (Rally®), and sulfur—all products that are commercially registered in California viticulture. Sulfur was dusted weekly while the three synthetic fungicide applications were made every other week, and the deficit irrigation treatment applied just one quarter of the water to the vines than they would normally have received from berry set to harvest (Hudson, 2012).

While it has been proven that fungicides can manage or control pathogen attacks in crops, there is less definitive research to show whether plant responses to fungicide applications, like the induction of specific phytochemicals, are increased or decreased with pesticide applications.

Vrcek et al. (2011) sought to find the effects of organic versus conventional viticulture practices on polyphenol contents in grapes. The specific pesticides used in the conventional

grape growing or the specific organic practices were not listed in Vrcek's study—the treatments were merely labeled "organic" or "conventional." However, the organic vineyards had been in compliance with Croatian regulations for certified organic management for over four years. The highest levels of gallic acid were found in the organic red wines, while the lowest levels were found in the conventional white wines. P-coumaric acid levels were also higher in the organic samples. Overall, this study suggested that phytochemical concentrations were higher in organic wines than conventional wines. With fewer pesticides, there are more pest attacks, and more plant defense chemicals produced. In this study, the authors use "organic" to mean a fruit without pesticide residues. However, there are other differences in management practices that might have contributed to these results, like the emphasis on cover crops and compost in organic farming, which leads to a slower release of nutrients and longer ripening period, in which the secondary metabolites are formed.

Ruiz-Garcia et al. (2012) conducted a study in which benzothiadiazole, an analog of salicylic acid, and methyl jasmonate, were applied to six year old Monastrell red wine grapevines. Salicylic acid and methyl jasmonate are regulators of key plant defense pathways. The study concluded that both compounds increased grape flavonoids, including anthocyanins, proanthocyanidins, and flavonols in both years of the study. Wine from the treated grapes in Ruiz-Garcia's study showed a higher total phenolic content when compared with wine that was made from the control grapes. Quercetin derivatives were the most important compound (quantitatively) that was identified in this grape variety. Since they show no persistence, don't leave residues, and increase phenolic content in grapes, these results piqued the authors' interests in using benzothiadiazole and methyl jasmonate as alternatives or complements to fungicide programs.

Romero et al. (2013) conducted a study on the effects of regulated deficit irrigation in a red wine variety, Monastrell. The study, which took place over three years, looked at three treatments: the control, which was a sustained regulated deficit irrigation regime that gave 40% of the crop evapotranspiration (ETc), an average of what most Monastrell grape growers were applying in Jumilla, Murcia vineyards—the region in Spain where this study took place. The first regulated deficit irrigation treatment irrigated at 30% ETc in the first two years and 20 % in the last year. The second regulated deficit irrigation treatment irrigated at 20% ETc in the first two years and 10% in the last year. From fruit set to veraison, or the onset of ripening, there was a cutoff in irrigation. Overall, there was an increase in the concentration of phenolic compounds in both regulated irrigation deficit treatments when compared to the control. More specifically, in 2010 and 2011, the first regulated deficit treatment berries showed more extractable anthocyanin content and polyphenols than the second regulated deficit treatment berries. All in all, when there was mild water stress early in the season and a moderate stress during pre- and post-veraison, there was an improvement in berry and wine quality.

Based on the findings in the studies discussed in these papers, it is clear that further research needs to be conducted to determine how factors such as fungicides and deficit irrigation affect the phenolic compound production in wine grapes.

Materials and Methods

Wine samples, Chardonnay and Cabernet Sauvignon, from the fungicide and deficit irrigation study at San Lucas in 2012 (Hudson 2012) were analyzed using a high pressure liquid chromatography (HPLC) machine at Cal Poly, San Luis Obispo. The HPLC is a liquid chromatography instrument from Shimadzu (LC-20AT). It is equipped with an auto-sampler, a

diode array detector, a prominence degasser, a communications bus module, and a column oven. There were two HPLC buffers: Solvent A and Solvent B. Solvent A was 0.1% trifluoroacetic acid, from the Sigma Chemical Company, in MilliQ water. The solvent was filtered through a bottletop filter. Solvent B was 0.1 percent trifluoroacetic acid dissolved in acetonitrile, from Fisher Scientific.

The standard samples were prepared in Solvent A. For gallic acid, 20 mg of gallic acid, also from Sigma Chemical Company, was dissolved in 100 mL of solvent, creating a 200 mg/L solution. Other standards, including quercetin glycoside, were prepared using 20 mg of the sample and diluting that with 50 mL of solvent, creating a 400 mg/L solution. Dilutions of the standards were created by combining the undiluted standards. For example, the coumaric/catechins sample was created by adding equal volumes of the undiluted catechins and coumaric acid, making the concentration now 200 mg/L for each compound. This was then diluted 1:2 with solvent A, making a 1:4 dilution (100 mg/L of each compound). This process was continued to make several dilution standards. The samples were then filtered into HPLC vials using 13 mm diameter, 0.22 micron syringe filters, from Bausch and Lomb (P. Rice, personal communication, January 17, 2014).

Three different peaks were studied among the fourteen Cabernet Sauvignon samples and two peaks were studied among the fifteen Chardonnay samples. In the Cabernet Sauvignon samples, one peak was studied at 531 nanometers (likely quercetin) and two peaks were studied at 369 nanometers. The retention times ranged from 37-41 minutes, 40-44 minutes, and 47-50 minutes respectively. In the Chardonnay samples, one peak was studied at 328 nanometers, with the retention times ranging from 20-22 minutes and the other peak was studied at 329 nanometers, with the retention times ranging from 23-24 minutes. The areas of those peaks,

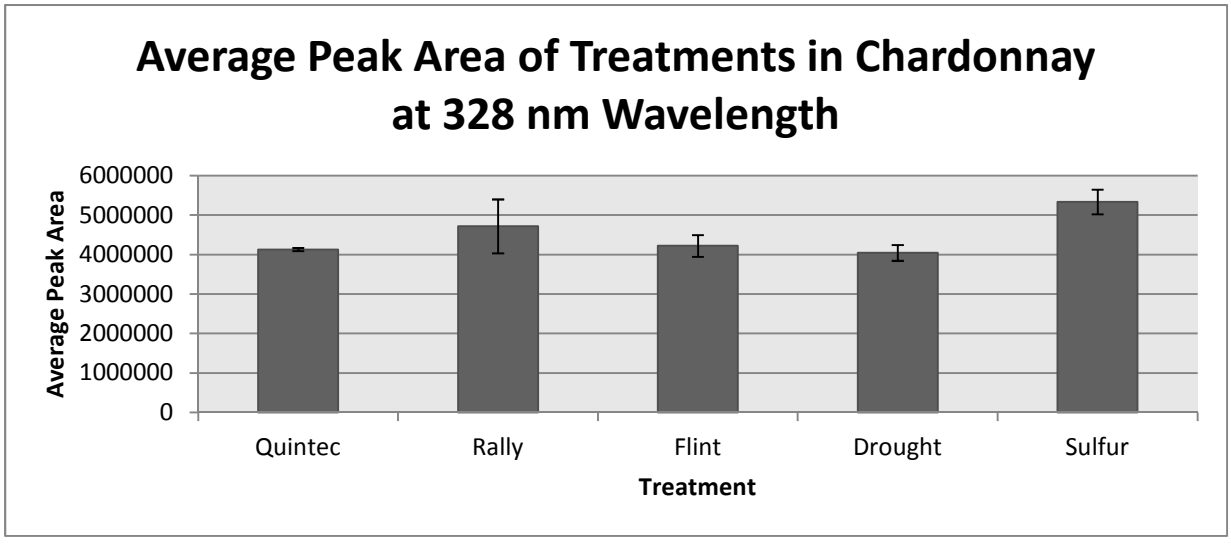
which are calculated from the HPLC, are then recorded to give us our "peak area" for that sample, which is the data that has been collected as part of this project. Data were analyzed by analysis of variance (ANOVA), using mean PA as the dependent variable and treatment and block as independent variables, and using p-value <0.05 as a level of significance. Mean separation was performed by Tukey's honestly significant difference test. PA values were log 10 transformed before running the analysis.

In the Cabernet Sauvignon samples, there are three replications for the Quintec®, Rally®, Flint® and drought treatments and two replications for the sulfur treatment. In the Chardonnay samples, there are three replications for each treatment, Quintec®, Rally®, Flint®, drought, and sulfur. The average peak areas of the replications were taken for each treatment and used in the statistical analysis.

Results

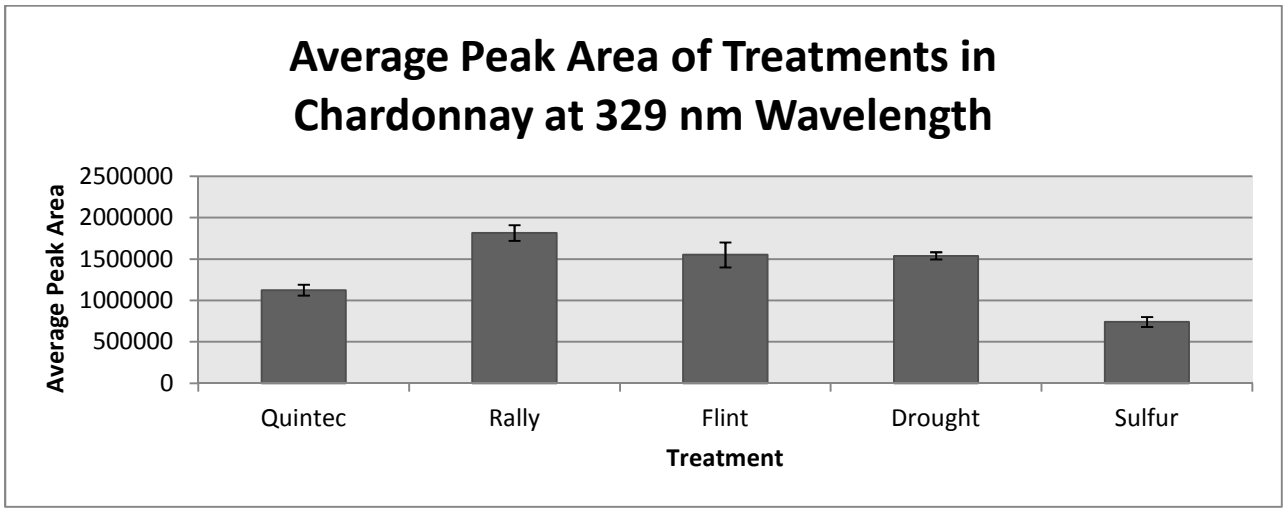
The following figures show the average peak areas of the data collected from the HPLC machine.

Figure 1. Average peak area of each treatment for the CH lambda 328 data.



The average peak area among the treatments ranged from 4,043,379 to 5,331,772. The drought treatment had the lowest peak area, while the sulfur treatment had the highest peak area. There was no significant difference found among the treatments.

Figure 2. Average peak area of each treatment for the CH lambda 329 data.



The average peak area among the treatments ranged from 741,299 to 1,815,119. The sulfur treatment had the lowest peak area and the Rally® fungicide treatment had the highest peak area. Since the p-value is less than 0.05, there was significant difference found among the treatments, as shown in Tables 1 and 2. The Quintec® treatment differed from the Rally® and sulfur treatments. The Rally® treatment differed from the Quintec® and sulfur treatments. The Flint® treatment differed from the sulfur treatment. The drought treatment differed from the sulfur treatment. The sulfur treatment differed from the Rally®, Flint®, drought, and Quintec® treatments.

Table 1. Summary of the ANOVA for the CH-lambda 329 data.

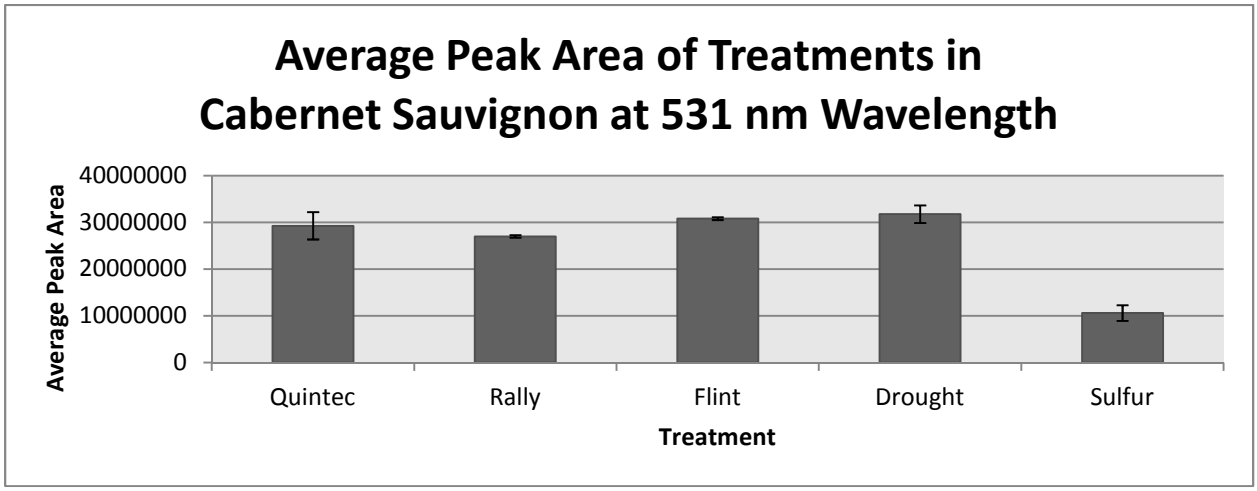
Variety	Lambda	RT	F	df	P
CH	329	23	20.19	4,8	0.003

Table 2. Mean separation analysis using Tukey's HSD for the CH lambda 329 data.

CH 329	Treatment	Tukey mean separation code
	Rally	a
	Flint	ab
	Drought	ab
	Quintec	b
	Sulfur	c

Each treatment that has the same letter means those treatments are not significantly different from one another. For example, all treatments with "a" included in the code are not statistically significantly different. If treatments have contrasting letters, like "ab" and "c," then that pair of treatments are significantly different from one another.

Figure 3. Average peak area of each treatment for the CS lambda 531 data.



The average peak area between the treatments ranged from 10,602,028 to 31,760,044. The sulfur treatment had the lowest peak area, while the drought treatment had the highest peak area. Since the p-value was less than 0.05, there was significant difference found among the treatments, as shown in Tables 3 and 4.

Table 3. Summary of the ANOVA for the CS-lambda 531 data.

Variety	Lambda	RT	F	df	P
CS	531	39	15.19	4,7	0.001

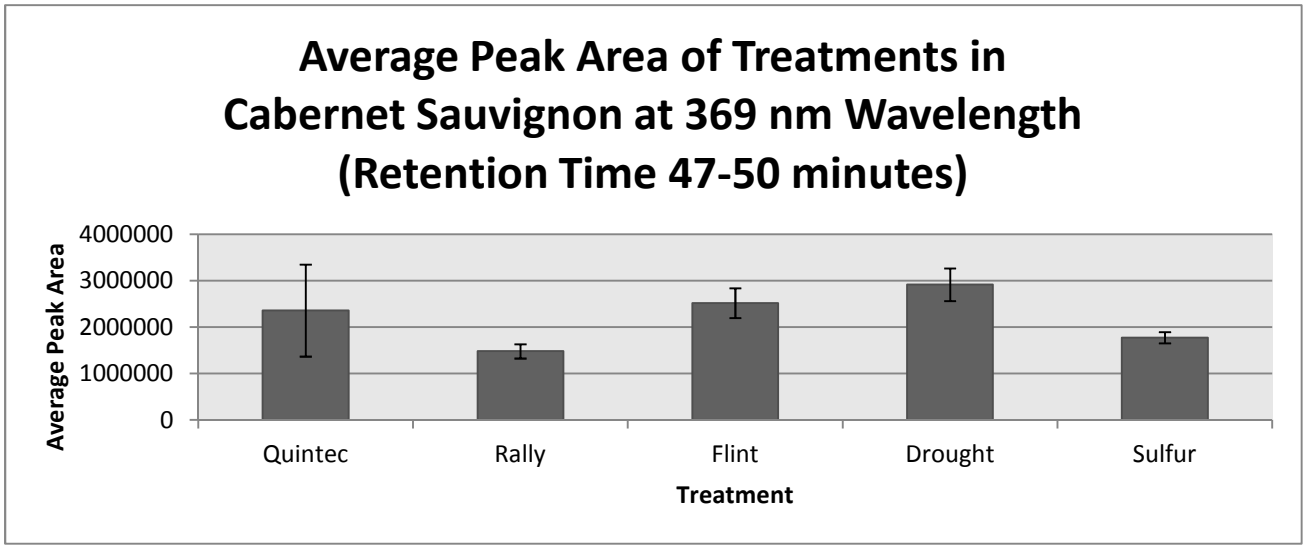
Table 4. Mean separation analysis using Tukey's HSD for the CS lambda 531 data.

CS 531	Treatment	Tukey mean separation code
	Quintec	b
	Rally	b
	Flint	b
	Drought	b
	Sulfur	a

Each treatment that has the same letter means those treatments are not significantly different from one another. For example, all treatments with "a" included in the code are not statistically significantly different. If treatments have contrasting letters, like "ab" and "c," then that pair of treatments are significantly different from one another.

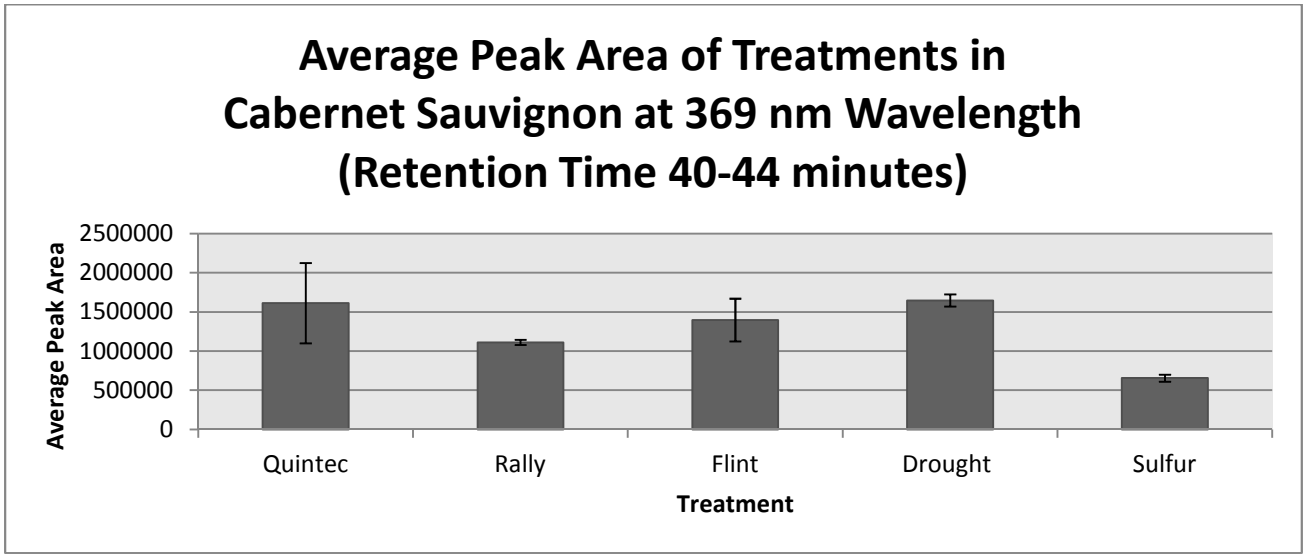
These codes mean that the Quintec®, Rally®, Flint®, and drought treatments (all labeled "b") are not significantly different from one another, but collectively, those treatments are significantly different from sulfur, which is the only treatment with a different code, "a".

Figure 4. Average peak area of each treatment for the CS lambda 369 (47-50 minute retention time range) data.



The average peak between the treatments ranged from 1,481,445 to 2,917,659. The Rally® treatment had the lowest peak area, while the drought treatment had the highest peak area. There was no significant difference among the treatments.

Figure 5. Average peak area of each treatment for the CS lambda 369 (40-44 minute retention time range) data.



The average peak areas ranged from 655,629 to 1,647,504. The sulfur treatment had the lowest peak area, while the drought treatment had the highest peak area. There was no significant difference among the treatments.

Discussion

Hudson's study (2012) found sulfur to have "the lowest average color reading for all treatments at all wavelengths." In the Cabernet Sauvignon (CS) 531 sample, sulfur also produced the lowest peak area, which was significantly smaller than the other peak areas for all other treatments. The compound at this peak is quercetin, which is an anthocyanin; anthocyanins are responsible for color in red wines. A speculation for this could relate to the fact that the addition of sulfur dioxide to the must bleaches color by forming a complex with anthocyanins in the red wine (Bisson, 2008). However, color of the wine wouldn't be permanently affected as the binding can be reversed, so even if this thought proved to be accurate in subsequent research, the

red wine may regain its original color later on. Therefore, it appears that the application of sulfur dust to the vines seems to inhibit or interfere with anthocyanin production or manifestation. As this wavelength is one that is responsible for color in red wines, the worst fungicide to use if we were looking for increased color in our wine is sulfur, while the four remaining treatments would be better as they produced larger peak areas. While the sulfur treatment in CS 369 (the lower of the two retention times) also produced the lowest peak area, this treatment was not found to be significantly different from the others.

In the CH 329 wavelength, the sulfur treatment produced a significantly smaller peak area compared to the other treatments. Similar to the speculations above, the sulfur may be binding to other compounds, essentially masking the discernibility of that phenolic at that specific wavelength. More specifically, bisulfite forms complexes with acetaldehyde and that binding restricts sensory characteristics, many of which come from phenolics. Reduced sensory characteristics could mean a lower concentration of phenolics, resulting in a smaller peak area, as shown in the graph. Low levels of acetaldehyde contribute positively toward aroma, while high levels result in poor smelling wines; higher levels come from wines that are fermented in the presence of sulfur dioxide (Liu & Pilone, 2000). While it is not definitive what phenolic compound is responsible for creating these peaks at this specific wavelength, we could infer that sulfur would be the worst fungicide to use if we were looking to increase that specific phenolic in our wine as it had the lowest peak area. The Rally®, Flint®, or the drought treatment would be the best fungicide to use if we were looking to increase that specific phenolic in our wine as the Rally® treatment produced the highest peak area, and was not significantly different from the Flint® or drought treatments.

Romero's study on regulated deficit irrigation in Spain could also be applied to our current study. Romero suggested that regulated deficit irrigation showed more extractable anthocyanin content compared to the control treatment. In the Cabernet Sauvignon 531 wavelength, which is a wavelength that is responsible for color (anthocyanins), the drought treatment produced the largest absolute peak area average, and although it was not significantly different from the fungicide treatments, it may suggest a similar outcome to Romero's findings. Furthermore, Romero had also stated that there was an increase in the concentration of phenolic compounds with the regulated deficit irrigation treatment. Both other wavelengths (369 with different retention times) for Cabernet Sauvignon showed that the drought treatment produced the largest absolute peak area average, suggesting that the drought treatment also produced the greatest concentration of phenolic compounds, though these results were not significantly different. However, it was noticed that the drought treatment did not produce the highest peak area for either of the wavelengths observed in the Chardonnay samples. A possible explanation for why the phenolics would be increased in Cabernet Sauvignon samples when under water stress but not in Chardonnay samples may have to do with the differences between making red wines and making white wines. Red wines are made with the grape skin, while white wines are not. During drought stress, the vine produces smaller berries, yielding a higher skin to juice ratio. Since some phenolics are located in the skin of grapes and red wines are made with the skins, it could make sense that only red wines show an increase in the concentration of phenolics.

Overall, the results of this study suggest that fungicides and deficit irrigation do influence the concentration of phenolics in wine. Further studies of sulfur's inhibitory effects on different compounds could be conducted in several more varieties of wine, beside Chardonnay and Cabernet Sauvignon, to illuminate these consequences to a larger group of growers. Subsequent

studies on drought stress at different times of the growing season on red wine varieties could explain to viticulturists the best times to withhold water from their vines in order to produce a better wine. With the information found in our study, hopefully viticulturists could make an educated decision of what fungicide should be applied in their vineyard, based upon what the pesticide could contribute to the phenolic profile of their wine.

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