Pennsylvania Wine Marketing and Research Program
Final Report

Project Title: Boosting polyfunctional thiols and other aroma compounds in white hybrid wines through foliar nitrogen and sulfur application.

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Project Overview and Key Objectives:
Nitrogen (N) is an essential mineral for plant growth, metabolism and health, and is commonly applied via soil and/or foliar treatments. Early season shoot growth is heavily dependent on reserve N and total nonstructural carbohydrates, and by the beginning of bloom, reserves are often depleted. Therefore, bloom through véraison and post-harvest periods are the two critical demand periods for vine nitrogen uptake, and consequently, the most efficacious time periods to supply nitrogen, mainly through soil application (Wolf 2008). However, these recommendations are mainly based on vegetative growth consideration rather than fruit yield and composition (in particular, yeast assimilable nitrogen; YAN). For example, a recent study conducted in Virginia showed that foliar application of nitrogen on Sauvignon blanc and Petit Manseng (*Vitis vinifera*) was more effective in increasing berry YAN than soil applied nitrogen (Moss 2015). Soil applied N was assimilated primarily into the vegetative components of the vine. Therefore, proper nitrogen management in the vineyard affects fermentation and, ultimately, wine quality, as adequate YAN in the grapes is critical to ensuring high quality fermentation (Leonardelli 2013).

In general, high N status in grapevines is associated with higher levels of glutathione and cysteinylated aroma precursors, including glutathione- and cysteine-linked precursors for the powerful polyfunctional thiols such as 3-mercaptohexanol (3MH), 3-mercaptohexyl acetate (3MHA), and 4-methyl-4-sulfanylpentan-2-one (4MSP) (Geffroy et al. 2017). These aroma compounds have exceedingly low aroma threshold values and contribute to varietal typicity in a number of cultivars, by imparting aromas of grapefruit, passionfruit, and blackcurrant. While Sauvignon blanc is most commonly associated with these beneficial varietal thiols, they are important to the aroma profiles of many other *Vitis vinifera* (e.g., Gewürztraminer, Colombard), hybrid (Cayuga White) and *Vitis labrusca* (Niagara) varieties (Dubourdieu & Tominaga 2009; Jeffrey 2016; Musumeci et al. 2015).

Recent work in Europe (Lacroux et al. 2008; Geffroy et al. 2017) and in the US (Kelly 2017) on various *Vitis vinifera* varieties has demonstrated that the concentrations of these important aroma compounds can be increased through foliar N and sulfur (S) spraying. Combining N with S led to a more pronounced effect than the N foliar spray alone, most likely due to synergistic adsorption of N in presence of S (Tea et al. 2007). N and S uptake in plants is intrinsically linked as both are important building blocks for cysteine, methionine, and glutathione.

In all of the above-mentioned studies, total N content, YAN and amino acid concentration in the juice, as well as glutathione and varietal thiols (3MH, 3MHA) in the wines, were observed to increase in foliar sprayed treatments compared to the untreated control, while the foliar treatments and the untreated control did not differ in canopy density, yield and juice Brix, pH and titratable acidity (TA). Evaluation of the treated wines (Lacroux et al. 2008; Geffroy et al. 2017) by wine experts revealed higher aroma intensities in grapefruit and tropical fruit while no reductive notes were perceived.

Taking all reports together, foliar spray applications of N and S present an interesting and potentially low-cost approach to enhancing wine quality through overexpression of important varietal thiols. We proposed a pilot experiment to test the applicability of foliar nitrogen and sulfur sprays on the white hybrid cultivar Traminette.

Traminette, a hybrid of Gewürztraminer and Seyval blanc, is grown all over the Commonwealth, and produces wines with similar characteristics to Gewürztraminer. It is growing in popularity and commercial significance (Vigna 2016), as it is well-suited to Pennsylvania's climate and its cold hardiness is superior than its Gewürztraminer parent (Reisch et al. 1996).

Similar in aroma to its Gewürztraminer parent, Traminette grapes and wines are characterized by high levels of the varietal terpenols linalool, geraniol and nerol that are linked to pleasant floral aromas and flavors in wine (Ji & Dami 2008; Skinkis et al. 2008). Traminette also contains polyfunctional thiols at similar levels to that of Gewürztraminer (Roland et al. 2011) and thus, presents an excellent cultivar candidate for our pilot experiment. The proposed interdisciplinary study described here aims to characterize the effects on viticultural, chemical and sensory properties of Traminette juice and wine, using (i) foliar urea N spray, (ii) foliar micronized S spray, (ii) foliar urea N and micronized S spray, and (iv) an untreated control.

Our specific **objectives** were to:

1. Assess the impact of N and S foliar application (either alone or in combination) at véraison on vine balance, yield components, and fruit chemistry.
2. Characterize the effectiveness of N and S foliar application (either alone or in combination) at véraison to impact aroma and flavor composition of finished wine, with a focus on important aroma compounds.
3. Determine the sensory impact and consumer acceptance of the wines made from treated versus control vines.
4. Provide growers and wine producers with recommendations for cost-effective means to boost polyfunctional thiols in Traminette and potentially other white hybrid cultivars

The proposed pilot study sought to expand and test recent findings in *Vitis vinifera* cultivars in a hybrid important to the Pennsylvania wine industry, with potentially high impacts to grape growers in Pennsylvania of not just Traminette, but also other hybrid and non-hybrid wine grape cultivars. It would enable grape growers to optimize wine quality through minimal and cost-effective viticultural interventions.

Final Methodology Used:
Field and wine evaluations were conducted over the 2018 growing season.

Experimental layout: A Traminette grower cooperator in Centre County was identified in January 2018 and his vineyard was used as the experiment site. In the spring 2018, the experiment was set up as a randomized complete block design with 4 replications per treatment, each comprising of 12 contiguous vines. Treatment replicates within the row were separated by 3 to 4 guard vines. To standardize the number of shoots per vine, shoots were thinned to 16 shoots per meter of cordon shortly after bud burst.

The treatments consisted of

1. Control with no nitrogen or sulfur applications other than sulfur applied by the grower as part of a disease management spray program
2. 15 kg/ha of urea N (Coron®, Helena Chemical Co., Collierville, TN) split in two equal foliar applications, the first at the onset of véraison and the second one week later
3. 5 kg/ha of micronized sulfur (Micro Sulf®, Nufarm Americas Inc., Burr Ridge, IL) in two split applications at the onset of véraison and one week later
4. 15 kg/ha of urea N and 5 kg/ha of micronized sulfur in two split applications at the onset of véraison and one week later.

Vegetative growth: To assess the impact of the treatments on canopy density and microclimate of the fruiting zone, point quadrat analysis and light availability in the cluster zone (EPQA) was measured before treatments application and again after treatment application, shortly before harvest. Pruning weight were collected during the winter season following treatments application. Vine crop load were estimated as Ravaz index (yield/pruning weight).

Vine nutrient status: Forty leaf petioles were sampled from each treatment replicate and analyzed for macro- and micronutrients concentration. Concurrently, leaf blades were used for determining total nitrogen concentration. Samples were collected at bloom (before treatments application) and again late in the summer, about 4 weeks after treatments applications.

Disease rating: Because N foliar application may increase fruit susceptibility to bunch rot disease, the severity (percentage of infected cluster area) and incidence (percentage of clusters infected) of *Botrytis* bunch rot were visually assessed using a scale from one to ten on 25 randomly selected clusters per each treatment replicate the day before harvest.

Yield component and fruit composition: Vines were harvested by hand one day prior to commercial harvest. The total number of clusters per vine were counted and the cumulative cluster weight per vine were measured. Five-hundred grams berry samples were randomly collected at harvest for berry chemistry analysis (total soluble solids, pH, TA) and average berry size was determined. Juice samples were also be analyzed for total yeast assimilable nitrogen (YAN) concentrations.

Research winemaking: Grapes were vinified in the Penn State Department of Food Science's Wet Pilot Plant facility using a standard winemaking protocol to eliminate potential sources of variation in the sensory profile of the wines. In brief, fruit was whole cluster pressed and the resulting juice was allowed to settle for 3 days at 4°C before being racked to glass fermentation vessels. All fermentations were performed in duplicate. Following fermentation, all wines were transferred and cold settled at 4°C for 48 h prior to racking off the lees. The wines were then

allowed to cold stabilize for an additional 14 days prior to racking and bottling with the addition of 30 mg/L SO_2 in 750 mL glass bottles with aluminum screw cap closures lined with foil. Basic wine chemistry (TA, pH, ethanol, free and total sulfur dioxide, residual sugar) was measured.

Juice and Wine compositional analyses: Volatile aroma profiles of the grape juices and wines were measured, using established methods that are optimized to capture important fermentation-derived and grape-derived aroma compounds. Terpenol and fermentation-derived (for wines) compounds were detected with HS-SPME-GC-MS, similar to (Hopfer et al. 2012; Hendrickson et al. 2016).

Wine sensory analysis: All proposed sensory tests were conducted at the Sensory Evaluation Center (SEC) at Penn State, using in a sip-and-spit protocol that was approved by Penn State's IRB. Ninety nine (99) wine consumers were recruited based on these criteria: (i) being of legal drinking age (between 21 and 65), (ii) regular wine consumption of white wine at least once a week. Using a two-stage testing protocol, consumers first rated overall liking of the four treatment wines (= Ctrl, N, S, N+S) on a 9-point hedonic scale, anchored with "Dislike extremely" on the left and "Like extremely" on the right hand side of the scale. After a brief break (10-15 min), they were presented with 6 sets for the triangle discrimination task, using all possible combinations of the 4 treatments (Ctrl vs. N; Ctrl vs. S; Ctrl vs. N+S; N vs. S; N vs. N+S; S vs. N+S). At the end, a brief questionnaire was collected to capture demographical, wine consumption and wine expertise data. All wines were randomized across the tests and consumers to control for carry-over effects (Lawless & Heymann 2010). Significant differences in consumer acceptability were evaluated by Analysis of Variance (ANOVA), treating wines as fixed and consumers as random factors. Potential consumer segmentation were evaluated by internal preference mapping (IPM), using demographic, consumption and expertise data as supplementary variables. Data from the discrimination test were analyzed for statistical significance using the binomial distribution (Lawless & Heymann 2010).

Key Findings & Results:
A summary of our key findings for Year 1 of the project follow.

Treatment Key:

- Treatment 1: Control: no foliar nitrogen (N) or sulfur (S) application other than regular S applications for disease management
- Treatment 2: Nitrogen (N): 15 kg/ha N – applied as two equal foliar applications around veraison
- Treatment 3: Sulfur (S): 5 kg/ha S (MicroSulf) – applied as two equal foliar applications around veraison
- Treatment 4: N + S: 15 kg/ha N + 5 kg/ha S– applied as two equal foliar applications

The above foliar treatments were applied on 8/6/18 and 8/15/18.

Leaf samples were collected before treatment application (7/31/18) to confirm that all vines had similar N concentration in plant tissue before foliar applications. A second leaf sampling was conducted on 9/14/19, approximately four weeks after treatments application. For each experimental unit we collected 40 leaves, 20 for each canopy side. Leaves of the same age (first fully expanded leaf from the shoot tip) were used for nitrogen analysis.

Table 1. Effects of nitrogen (N) or/and sulfur (S) application on N concentration in the leaf blade and petiole and on yeast assimilable nitrogen (YAN) in the juice. Leaves were collected four weeks after the second N and S application; YAN was measured at harvest.

Treatment	N Leaf blade (%)	N Leaf petiole (%)	YAN juice (mg N/L)
Control	3.44	1.61	314.8
N	3.49	1.66	415.5
S	3.42	1.61	337.3
N + S	3.33	1.63	246.7
P-value	*0.316*	*0.423*	*0.209*

Table 2. Effects of nitrogen (N) or/and sulfur (S) application on yield components and juice chemistry at harvest 2018.

Treatment	Yield (kg/vine)	Clusters /vine	Cluster weight (g)	Berry weight (g)	TSS (Brix)	TA (g/L)	pH
Control	4.07	19.6	211.4	2.00	17.1	9.85	3.42
N	3.70	19.7	192.2	1.90	16.1	10.01	3.48
S	4.01	22.5	172.0	1.98	16.0	10.53	3.38
N + S	4.95	23.4	204.1	1.89	16.6	9.75	3.41
P-value	*0.418*	*0.182*	*0.420*	*0.455*	*0.178*	*0.277*	*0.544*

Fruit was hand-harvested on 9/28/18 and was sorted both in the field and at the winery to remove damaged and/or rotten berries. Given the abnormal growing conditions in 2018 (i.e., record rainfall and, in particular, an extremely wet post-veraison period), virtually every cluster in the control and treatment blocks were afflicted with at least some level of rot. Furthermore, sugar accumulation was lower than what was expected given a late September harvest date, with harvest soluble solids levels recorded at ca. 16 Brix. The potential confounding effects of these conditions on the study's results (and the conclusions that can be drawn) are discussed below.

Table 3. Juice chemistry results.

Treatment	Brix	pH	TA	YAN
Control	16.0	3.18 @ 20.4 C	9.375	282.46
N	16.6	3.17 @ 20.4 C	9.288	293.27
S	16.0	3.17 @ 20.2 C	9.235	232.9[1]
N + S	16.2	3.19 @ 20.4 C	9.455	291.4

Standard white winemaking protocols were followed according to the methods described above, and the resulting juices were sulfited (30 mg/L SO_2) and treated with 1.5 mL/HL CinnFree (enzyme). Following juice settling (9/29/18), juices were chaptalized with sucrose to 22 Brix (final) and inoculated with VIN13. The final volumes were 13.82 L of juice per fermentation replicate for Treatments 1 (Control), 3 (S), and 4 (N+S); the final volume for Treatment 2 (N) was 12.3 L per fermentation replicate. Once the wines reached zero Brix (as confirmed by enzymatic analysis), they were allowed to settle before being racking and before a final sulfite adjustment was made. The wines were transferred to 750 mL capacity bottles under gaseous nitrogen blanket and sealed using ROTE (screw cap) closures. Sample aliquots were taken at this time, which were transferred to 50 mL capacity centrifuge tubes and frozen at -80 C until volatiles analysis could be performed. Wine samples stored in 750 mL glass bottles were maintained at 15 C until sensory analysis could be performed.

[1] YAN was adjusted to a final concentration of 250 mg N/L

Analysis of Wine Volatiles by GC-MS

The volatile fraction of each wine sample was chromatographically separated and analyzed by headspace GC-MS according to the methodology described above. A representative GC-MS chromatogram is shown in Figure 1.

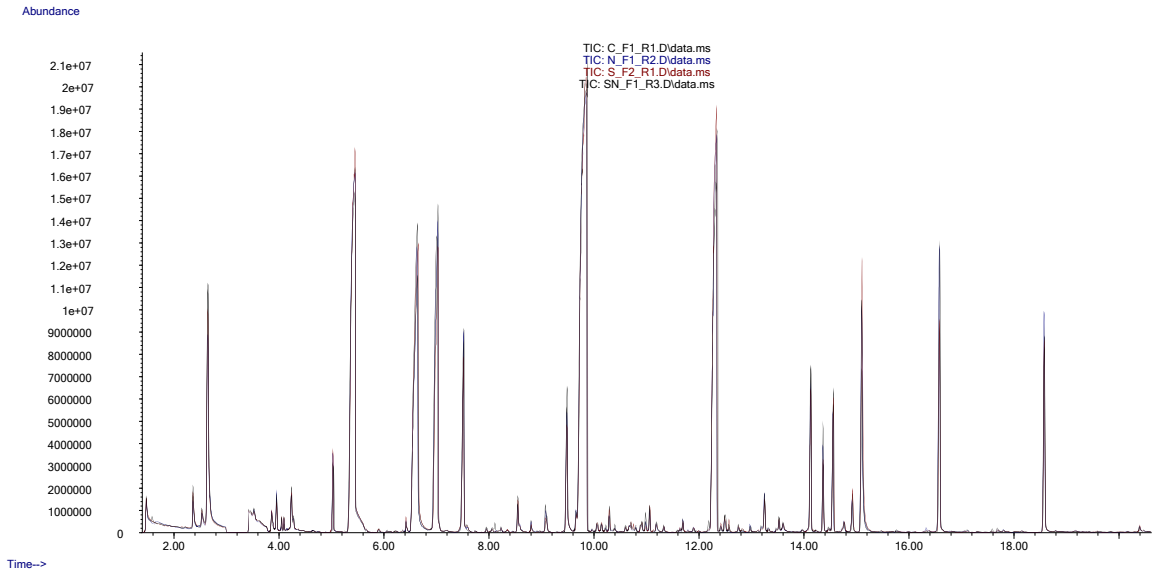

Figure 1. Representative chromatogram

Particular attention was paid to both undesirable and desirable aroma-active, non-terpenoid, volatile compounds in the control and treatment wines (i.e., ethyl acetate, isoamyl acetate, isoamyl alcohol, ethyl hexanoate, hexyl acetate, ethyl octanoate, ethyl decanoate, 2,phenylethyl acetate, hexanoic acid, ethyl dodecanoate, 2-phenylethyl alcohol, octanoic acid, and decanoic acid). The relative retention times of these analytes, as well as their relative abundances, are shown in Figure 2 for the four wines (C, N, S, S+N). We noted no significant differences between the control and treatment wines in terms of any of these aroma active compounds, suggesting that the foliar spray application treatments had no influence on the presence/absence or final concentrations of these volatile compounds of interest.

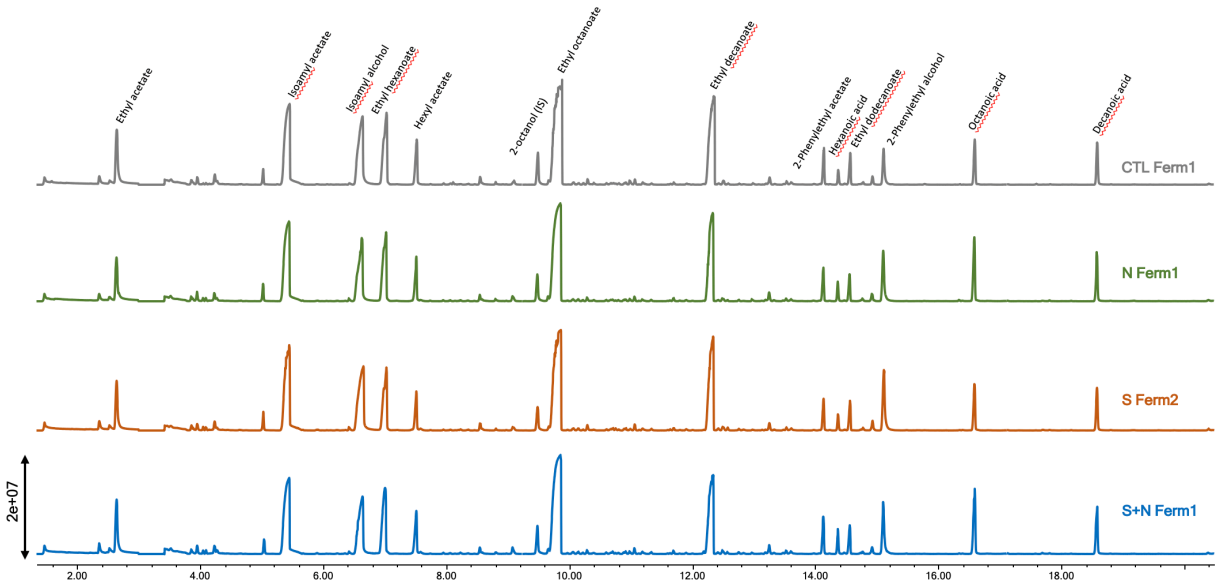

Figure 2. Gas chromatogram showing mass spectrum detection of key aroma active volatile compounds in control and treatment wines. All chromatograms are scaled to the same abundance intensity; shown are total ion current (TIC).

Terpenoids are extremely important to the overall aroma quality of wines, even when present at exceedingly low concentrations due to the low sensory thresholds in humans. Therefore, we profiled the aroma fraction of the four wines (C, N, S, S+N) specifically for a panel of key terpenoids, which include beta-myrcene, linalool butanoate, linalool, neryl acetate and geranyl acetate). As was the case with the non-terpenoid fraction (Figure 2), we observed no significant differences between any of the treatment wines versus the control wine in terms of volatile terpenoids. This, again, suggests that the foliar spray interventions had no bearing on aroma active terpenoids.

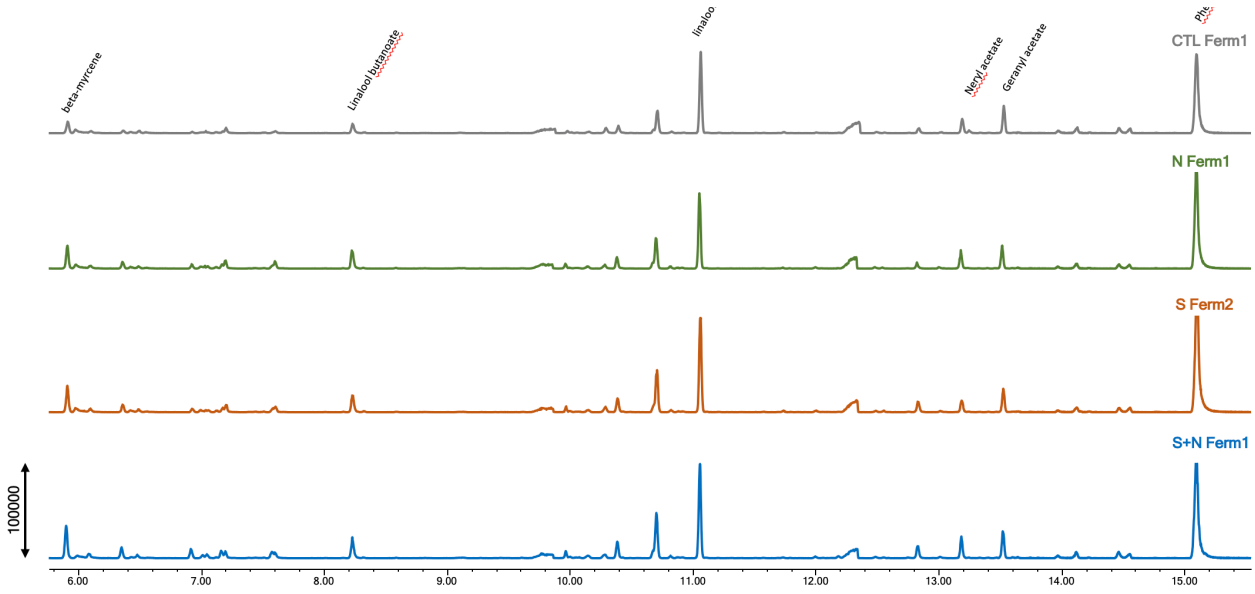

Figure 3. EIC for *m/z* 93 to highlight terpenoids (key aroma active compounds of interest). All chromatograms are scaled to the same abundance intensity.

Finally, the wines were analyzed in terms of their varietal (polyfunctional) thiol content, specifically the key thiols 3-mercaptohexanol (3MH), 3-mercaptohexyl acetate (3MHA), and 4-methyl-4-sulfanylpentan-2-one (4MSP). We were unable to detect 3MH, 3MHA or 4-MSP in any of the wines (data not shown). This might be due to the fact that these compounds were present below their lower limit of detection (a likely scenario given how low their typical concentrations are in wines). Another possibility is that these compounds were present at some point in the fruit but were subsequently lost due to enzymatic or non-enzymatic degradation (oxidation) reactions in the vineyard because of the poor quality of the fruit at harvest. Polyfunctional thiols are exceedingly labile to oxidation reactions, as discussed above.

Sensory Analysis of Wine Samples

The control wine and foliar treated wines (N, S, N+S) were analyzed by human sensory panelists according to the protocol described above. In brief, 99 wine consumers were presented with 6 sets for the triangle discrimination task, using all possible combinations of the 4 treatments (Ctrl vs. N; Ctrl vs. S; Ctrl vs. N+S; N vs. S; N vs. N+S; S vs. N+S). The panel, as a whole, was unable to correctly discriminate between the control wine and any of the treatment wines (Figure 4). These results corroborate the instrumental analysis of volatiles (by GC-MS) discussed above, and again suggest that none of the foliar applications were able to affect final wine quality.

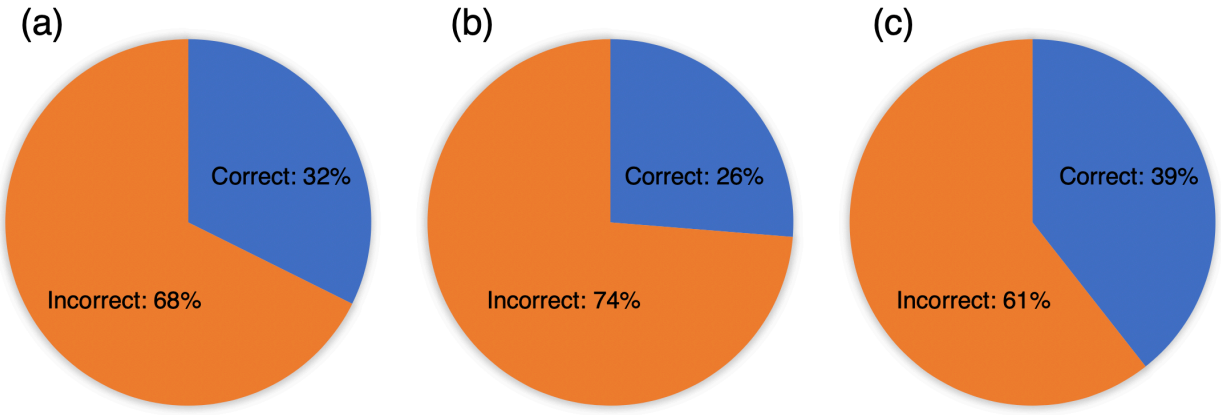

Figure 4: Ability of human sensory panelists to correctly or incorrectly identify a wine made from foliar spray treated wine compared to a control wine. Figure (a) represents Control vs. Nitrogen (N) wines; Figure (b) represents Control vs. Sulfur (S) wines; Figure (c) represents Control vs. Nitrogen + Sulfur (N+S) wines. Total number of observations (participants) = 99.

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