YEAST ASSIMIABLE NITROGEN IN GRAPES AND ITS RELATIONSHIP TO THE ORIGIN OF AROMA COMPOUNDS

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Mark Andrew Nisbet

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Mark Andrew Nisbet, Ph. D.

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Wine is a complex mixture of over 100 volatile compounds produced via various biochemical pathways, some in the grapes, and others de novo through yeast metabolism. Nitrogen is associated with the production of alcohols and esters during fermentation, and is often the limiting metabolic factor for fermentation. The biochemical pathways utilized by yeast to produce aroma compounds are affected by the concentration and source of nitrogen. This dissertation investigates the development of nitrogenous compounds in grape berries during ripening, methods to predict concentrations before harvest, the use of isotope ratio mass spectrometry (IRMS) to investigate the origins of fermentative aroma compounds, and explores the way taste physiology affects wine perception.

In the first study of yeast assimilable nitrogen, 60 sites across the Finger Lakes region of New York State were sampled during a three-year period (2010 through 2012) and regression models were developed to predict YAN two weeks before harvest. The second study on YAN expanded the investigation to YAN in grape berries from veraison to harvest in Cabernet Franc, Chardonnay, Merlot, Noiret, Pinot noir, Riesling, and Traminette cultivars across New York State. Chronic deficiency (YAN concentrations less than 140 mg/L) was observed in Cabernet Franc, Riesling, and Traminette, while Chardonnay and Pinot noir were consistently above 200 mg/L. Population distributions and regression models developed from this work can be used to decrease the amount of supplemental nitrogen added to the must prophylactically, while minimizing the chance for over or under supplementation.

The next portion of the research investigated the development of an IRMS method to determine what portion of aroma compounds are produced from yeast metabolism of sugar verses other grape precursors. The study demonstrated that most of the aroma compounds derived their carbon from sugar metabolism, while 1-hexanol derived most of its carbon from grape sources other than hexoses.

Finally, in a preliminary experiment, inexperienced PROP tasters reported being significantly less confident when choosing a wine than non-tasters. However, a follow-up experiment with more experienced wine consumers showed no significant differences in confidence or influence of external cues on panelists with different taste phenotypes.

BIOGRAPHICAL SKETCH

Mark Andrew Nisbet grew up in Leland, IL - population 970. Growing up surrounded by 400 acres of corn and soybeans, and being involved in short-lived entrepreneurial endeavors into chicken farming and sheep herding, he was never far from the food supply. During summer stints working at Glenora Wine Cellars in Dundee, NY Mark developed an appreciation for another aspect of food science, enology. Mark graduated from the University of Illinois Urbana-Champaign with a Bachelors of Science in biological engineering in 2003. He spent the following six years honing his skills as a food scientist at the International Food Network where he developed new products and innovations for clients throughout the food industry. Mark began as a doctoral student at Cornell University in Food Science and Technology in 2010.

I dedicate this work to my family.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The transformation of grape juice into wine by *Saccharomyces cerevisiae* is a complex biochemical process that produces an equally complex beverage. Some wine aromas can be found in the grape, others arise during fermentation, and some may originate from post-fermentation treatments, such as aging in oak barrels (Garde-Cerdán and Ancín-Azpilicueta 2006). The fermentative aromas, including esters, fusel alcohols, acids, and volatile sulfur compounds, arise from multiple pathways involving the metabolism of carbon, nitrogen, and sulfur substrates (Ugliano and Henschke 2009). Nitrogen is often the limiting metabolic factor, and can influence the type and quantity of aromas produced (Cantarelli 1957, Salmon and Barre 1998). While a better understanding of nitrogen accumulation in grapes and the biochemical pathways involved in production of fermentative aromas may help optimize wine quality, consumers' perception of wine does not depend solely on its intrinsic properties. Perception of smellaromas and tastes are also influenced by differences in taste physiology, resulting in variable gustatory responses (Tepper 2008), and external cues such as packaging, labeling and the opinions of others (Wansink et al. 2007).

Nitrogen Accumulation and Prediction

Nitrogen is an important component in grape must and is frequently the limiting metabolic factor during fermentation (Cantarelli 1957). Grape must contains a variety of nitrogenous compounds, including ammonia, free amino acids (AA), peptides, and proteins, but not all of these forms can be assimilated by yeast (Bell et al. 1979). Primary amino nitrogen (PAN) and ammonia (AMM), both used by yeast, are known collectively as yeast assimilable nitrogen (YAN) (Bell and Henschke 2005). Proline, a secondary amine and the only proteinogenic amino acid not

assimilable by yeast under anaerobic conditions, is not included in PAN measurements (Salmon and Barre 1998). Nitrogen deficiencies can lead to stuck or sluggish fermentations and subsequent production of hydrogen sulfide (Ugliano et al. 2009, Acree et al. 1972, Vilanova et al. 2007, Vos and Gray 1979). It is generally accepted that a YAN concentration of 140 mg/L is the minimum required for healthy fermentation (Bely et al. 1990), and 400 mg/L has been found to be the maximum amount consumed by yeast (Bisson and Butzke 2000). Concentrations above 400 mg/L may result in residual nitrogen post-fermentation, subsequent microbial instability, and spoilage (Bell and Henschke 2005).

The concentration of YAN in grapes at harvest is highly variable, cultivar dependent, and is often below the minimum concentration of 140mg/L (Stewart 2013, Bell and Henschke 2005, Hilbert et al. 2003). Supplementation with commercial yeast nutrients is a common means of avoiding fermentation problems associated with nitrogen deficiency, but sound supplementation strategies require an estimate of the initial concentration of YAN in the grape must. Determining YAN concentrations requires specialized reagents and equipment (Gump et al. 2002), which may prevent wineries from performing this analysis in-house. Further, many winemakers do not have time to send samples to external analytical laboratories and wait for results. For this reason, many winemakers make prophylactic nitrogen additions without knowing their initial YAN concentration, which may lead to insufficient or excess YAN. This research aims to better understand the accumulation of YAN during berry ripening and estimate harvest YAN using regression models and population distributions. Even estimates of YAN with-in ±50 mg/L would greatly improve the ability to make supplementation decisions and decrease the risk for nitrogen deficiency or excess.

Using Isotope Ratio Mass Spectrometry to Identify the Origin of Fermentative Aromas

Fermentative aroma compounds such as fusel alcohols, esters, and acids may arise from multiple biochemical pathways. Although the possible routes for their formation are generally well established, the relative importance of the different pathways are not established. One example is the role of amino acids in the origin of fusel alcohols and esters. In 1907 Ehrlich first described the catabolism of amino acids by yeast during fermentation to produce fusel alcohols (Ehrlich 1907). However, yeast may also produce fusel alcohols through sugar metabolism (Ugliano and Henschke 2009). Garde-Cedran and Ancin-Azpilicueta (2008) concluded that addition of amino acids to must favors the formation of volatile compounds in wine. However it is still unclear if the amino acids are used directly as aroma precursors or if the additional nitrogen is encouraging increased *de novo* synthesis of aroma compounds. Currently, the relative contribution of each pathway is poorly understood.

In complex biochemical systems, stable isotopes have been used to trace the pathway of precursor compounds and determine their contribution to end products. In food chemistry tracers have been used to study precursors of Maillard reaction products (Schieberle 2005). In wine, SIDA was utilized to study the formation of branched chain fatty acid ethyl esters (Diaz-Maroto et al. 2005). Quantitative enrichment can be used to study compounds present at trace levels, but when precursors are present in macro quantities, as hexose concentrations are in wine, quantitative enrichment is impractical due to the cost of stable isotope tracers and the potential perturbation to the system physiology. High precision isotope ratio mass spectrometry (IRMS) is a technique that has been used across a variety of disciplines to determine very small differences in the isotope distribution of materials, thereby requiring much lower levels of isotope tracer (Asche et al. 2003).

This work provides the first use of an IRMS method to determine the contribution of sugar to the production of fermentative aroma compounds. Uniformly labeled (U-^{13}C) glucose was added to grape must prior to fermentation so that aroma compounds derived from hexose metabolism would carry the label. By measuring the atom percent excess of ^{13}C in secondary metabolites, it is possible to determine the contribution of hexose to the amount of carbon in an aroma compound.

Influence of Taste Phenotypes and External Cues on Wine Preference

Expectations can influence the perceptions of the foods and beverages we consume. It is not known, however, how taste physiology may interact with these external cues to influence liking of wine. For decades sensitivity to 6-n-propylthiouracil (PROP) has been known to follow a Mendelian inheritance pattern, with distinct phenotypes recently linked to expression polymorphisms of the TAS2R38 bitter receptor gene (Hayes and Keast 2011, Kim et al. 2003). Based on response to bitter stimuli (PROP), phenotypic differences have been used to classify individuals into three separate taste categories: supertasters, tasters, and non-tasters. More recently, however, measures independent of PROP have been utilized to identify individuals with heightened taste response. These include irritant bitter tasting (iBT) and thermal tasting (TT) (Bajec and Pickering 2008, Green and Hayes 2003).

Differential responses to oral stimuli have been shown to correlate to consumption of certain foods, such as cruciferous vegetables and alcoholic beverages (Duffy et al. 2010, Tepper 1998, Hayes et al. 2011). PROP supertasters, for example, have been found to perceive red wines as more bitter and irritating, and supertasters are hypothesized be more likely avoid wine styles that are high in bitter and astringent compounds (Pickering et al. 2004, Duffy et al. 2004). It has been hypothesized by Hanni and Utermohlen (2011) that because wine styles touted by experts

are often high in bitter and astringent compounds, consumers with aversions to these compounds will exhibit decreased wine consumption and have lower confidence when selecting a wine. Given that PROP tasters have aversions to specific foods, including red wine, we aimed to determine whether this population is differentially influenced by external cues.

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CHAPTER 2

PREHARVEST PREDICTION OF YEAST ASSIMILABLE NITROGEN IN FINGER LAKES RIESLING USING LINEAR AND MULTIVARIATE MODELING

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Abstract: A three-year study was conducted to determine if regression models could be developed to predict yeast assimilable nitrogen (YAN) prior to harvest, using Riesling in the New York Finger Lakes region as a model. Berry samples were taken from 62 commercial Riesling vineyards around the Finger Lakes at three time points: veraison, two weeks prior to harvest, and harvest. Samples were measured for berry weight, Brix, pH, titratable acidity, ammonia (AMM), primary amino nitrogen (PAN), and yeast assimilable nitrogen (YAN). The average YAN concentration at harvest was 91.8 mg/L, and there were no significant differences in harvest YAN concentration among years. Linear regression models created using preharvest YAN measurements (p <0.05) had a cross-validated R^2 (Q^2) of 70%. Models using only preharvest AMM had less predictive power (Q^2 = 63%), but may allow winemakers more analytical flexibility than those requiring complete YAN measurements. Models created using multiple linear regression (MLR) were also developed, and provided better predictive power, with a Q^2 of 74%. Finally, a multivariate approach using partial least squares regression (PLSR) was used to create models with the highest predictive power, with a Q^2 of 74%. The additional analysis required to obtain values for additional prediction variables may limit the practicality of MLR and PLS approaches. Because many winemakers are not able or willing to perform YAN measurements during the busy time of harvest, the development these regression models as predictive tools may allow winemakers to use preharvest analysis to calculate accurate additions

of supplemental nitrogen, allowing them to use less supplemental nitrogen and avoid the excesses that may result from prophylactic additions.

Introduction

When *Saccharomyces cerevisiae* ferments grape juice into wine, nitrogen is required to produce yeast biomass (Kunkee 1991). Grape must contains a variety of nitrogenous compounds including ammonia, free amino acids (AA), peptides, and proteins, but not all can be assimilated by yeast (Bell et al. 1979). Primary amino nitrogen (PAN) and ammonia (AMM), both used by yeast, are known collectively as yeast assimilable nitrogen (YAN) (Bell and Henschke 2005). Proline, a secondary amine and the only amino acid not assimilable by yeast under anaerobic conditions, is not included in PAN measurements (Salmon and Barre 1998).

YAN concentration in grape must is highly variable (Butzke 1998, Gockowiak and Henschke 1992, Hagen et al. 2008), and in Riesling grapes is often low (Stines et al. 2000). As nitrogen is often the limiting metabolic factor determining fermentation rate (Cantarelli 1957), its deficiency can lead to stuck or sluggish fermentations and the production of volatile sulfur off-aromas (Acree et al. 1972, Ugliano et al. 2009, Vilanova et al. 2007, Vos and Gray 1979). As such, many winemakers supplement their must to ensure healthy fermentations. The type and quantity of nitrogen supplementation can have significant impact on the concentration of volatile compounds in the finished wine (Salmon and Barre 1998). Bisson and Butzke (2000) found that when excess nitrogen is present the maxium amount consumed by yeast during fermentation is 400 mg N/L. Residual nitrogen from excessive supplementation can lead to microbial instability and subsequent spoilage defects (Bell and Henschke 2005). Further, high nitrogen levels can lead to the formation of ethyl carbamate, a known carcinogen, and biogenic amines, which can cause headaches and respiratory or gastrointestinal distress in susceptible individuals (Daudt et

al. 1992, Monteiro et al. 1989, Bach et al. 2011, Jansen et al. 2003). Although recommendations vary, it is generally thought that a nitrogen concentration of 140 mg N/L is the minimum needed to avoid fermentation difficulties (Bell, and Henschke 2005; Bely, Sablayrolles and Barre 1991). Bisson and Butzke (2000) identify the optimum level of YAN for must at 21 Brix to be 200 mg/L.

Sound supplementation strategies begin with understanding the initial concentration of YAN in the grape must. Determining YAN concentrations in wine requires specialized reagents and equipment (Gump et al. 2002), which may prevent wineries from performing this analysis in-house. Further, many winemakers do not have time to send samples to external analytical laboratories and wait for results. For this reason, many winemakers make prophylactic nitrogen additions without knowing their initial YAN concentration, which may lead to insufficient or excess YAN.

One strategy to facilitate winery YAN measurement is to develop predictive methods based on preharvest analyses. While previous studies have determined YAN concentrations of grape cultivars grown in various world regions, most of these survey studies focused on concentrations at harvest. The objective of this work is to determine whether preharvest measurements of grape berry chemistry can be used to develop statistically significant models that predict YAN at harvest. The amino acid concentration in grape must has been shown to increase during berry ripening, but then plateau or decrease slightly prior to harvest, depending on cultivar (Hilbert et al. 2003, Hernández-Orte et al. 1999). Post-veraison increases in amino acids in *Vitis vinifera* varieties are caused largely by increased proline concentrations (Stines et al. 2000), while AMM concentration decreases throughout ripening (Bell and Henschke 2005). If these metabolic changes are cultivar dependent, it may be possible to develop models based on

YAN concentrations measured two weeks preharvest to estimate nitrogen status at harvest. Such a tool will ease the time constraints for YAN analysis, and allow winemakers to develop supplementation strategies based on reliable analytical methods.

Materials and Methods

Experimental Design. This survey comprised sixty-two commercial Riesling vineyard sites in New York State sampled annually over a 3-year period from 2010 to 2012. The sampling area at each site was defined as twelve vines per row in two adjacent rows for a sample unit of 24 vines, and the same vines were sampled for all three years. Sample sites were selected with input from vineyard managers to capture a range of vine vigor and soil types; sites were designated by the vineyard managers as high vigor, low vigor, or unassigned. Vine management was performed by vineyard managers according to their own best practices.

Sample Collection and Processing. Grape berry samples were collected at three time points: veraison, two weeks prior to harvest, and harvest. Preharvest and harvest dates were determined by consultation with vineyard managers. All samples were collected during a three-day window, and the interval between preharvest and harvest sampling was 14 days ± 2 days. In 2010, 2011, and 2012 there were 6, 21, and 12 sites, respectively that were harvested before samples were collected, making data unavailable for that site and year. Each two hundred-berry sample was weighed on an Ohaus Pioneer PA3102 scale (Ohaus Corp. Pine Bluff, NJ), accurate to 0.01 g, to obtain fresh berry weight. Berries were then crushed immediately using a Stomacher® 400 paddle blender (Seward Laboratory Systems, Port Saint Lucie, FL) at 120 RPM for 60 seconds. Post stomaching, 50 mL of must was decanted from macerated berries for analysis.

Cluster counts per vine were recorded during preharvest sampling on two-panel sections (6 to 12 vines). At harvest 25 clusters were collected and weighed on a Sartorius 3807 MP81 scale (Sartorius Corporation, Bohemia, NY) accurate to 1g. Cluster counts per vine and average cluster weights were used to estimate crop yield per vine for each sample site.

Grape Berry Chemistry. YAN is comprised of AMM and PAN, which must be analyzed individually. A 2mL aliquot was drawn from the 50mL juice sample, placed in a microfuge tube and centrifuged at 12000 x *g* in an Eppendorf 5415C (Brinkmann Instruments, Westbury, NY) for 2 min prior to nitrogen assay. A Chemwell 2910 Multianalyzer (Unitech Scientific, Hawaiian Gardens, CA) was used to rapidly test samples. AMM was determined by the glutamate dehydrogenase (GDH) catalyzed condensation of ammonia and alpha ketoglutarate (ak-G) and simultaneous oxidation of nicotinamide adenine dinucleotide (NADH) (Ough 1969). The oxidation of NADH results in a decrease in absorbance at 340nm, which can be quantified by spectrophotometry(Unitech Scientific, Ammonia Extended Range UniTAB, 2007). PAN is determined by derivatization of primary amino groups by o-phthaldialdehyde and N-acetyl-l-cysteine (OPA/NAC) to form isoindoles, which are detected spectrophotometrically at 340 nm(Dukes and Butzke 1998) (Unitech Scientific, Primary Amino Nitrogen UniTAB, 2007).

Soluble solids (Brix) were measured using a digital refractometer (model 30016, Sper Scientific, Scottsdale, AZ) with temperature correction. Titratable acidity was measured with an auto-titrator (Titrino 798, Metrohm, Riverview, FL) and expressed as tartaric acid equivalents. pH was measured with an Accumet Excel XL 25 pH meter (Fisher Scientific, Waltham, MA) and an ion selective probe (Fischer Scientific).

Environmental Factors. Soil samples were collected from each site after harvest in 2010 and analyzed by the Cornell Nutrient Analysis Laboratory for standard fertility measurements

and soil health indicators, including % moisture, potassium, magnesium, calcium, iron, aluminum, manganese, zinc, soil pH, buffering capacity, organic matter, active carbon, mineralizable nitrogen, and aggregate stability.

Statistical Analysis Methods. Each sample site had 21 measures of fruit chemistry, namely, 3 sample points (veraison, per-harvest, and harvest) x 7 measurements (Berry wt., Brix, pH, TA, AMM, PAN, YAN) plus measures of clusters/vine, average cluster weight, yield/vine collected at harvest and a categorical measure of site vigor, totaling 25 potential regression coefficients. Additionally, data from 2010 included 14 measures of soil health. All data analysis was carried out using Minitab 16 (Minitab, Reading, MA) statistical analysis software.

One-way analysis of variance (ANOVA) was used to assess differences in berry chemistry values by year. Tukey's method was used post hoc to separate means at the 5% significance level.

A probability plot was used to evaluate the fit of a distribution to the harvest YAN data and estimate percentiles. Suitable distributions were selected by assessing the fit using the criteria of having a p-value < 0.05 and the lowest Anderson-Darling (AD) statistic.

Three approaches to regression modeling were used in this study to predict harvest YAN concentrations. Linear models related a single predictor variable to harvest YAN concentration. Multiple linear regression (MLR) models related many predictor variables simultaneously to harvest YAN. Finally, factor analysis and partial least squares regression (PLSR) summarized the covariance structure of the data and used predictor variables to create latent variables to relate to harvest YAN.

Linear Regression. The regression function was used to create linear regression models relating YAN at harvest to preharvest measures of AMM, PAN, and YAN. Individual models

were created for each year, and for the combination of all three years. Additionally, in 2011 and 2012, YAN measurements from the previous year were used to predict YAN.

Multiple Linear Regression. More complex models were created using stepwise multiple linear regression (MLR) analysis using 21 potential predictor variables that included measures of fruit chemistry at veraison and preharvest plus harvest values for berry weight, brix, pH, and TA at harvest (14 additional soil health indicators in were also used in 2010). At each step, coefficients could be added or removed based on their P-value using an α of 0.1 as the cut-off to add or remove coefficients. Models selection was determined by the lowest predicted residual sum of squares (PRESS), and additionally leave-one-out-cross-validation (LOOCV) was utilized to assess predictive power of the model. Models were created for each year individually and combined.

Factor Analysis and Partial Least Squares Regression. Factor analysis was used to summarize the covariance structure of the data. Principal components were used to extract factors and Varimax rotation was used to orthogonally rotate the initial solution. The first two factors were plotted to visualize the covariance structure.

Partial least squares regression (PLSR) analysis was used to model the YAN concentration in grapes at harvest from individual sites. For model building, all potential predictor variables (35 in 2010; 21 in 2011, 2012, and multi-year) were used to create an initial model of harvest YAN from individual sites (55 in 2010, 40 in 2011, 50 in 2012, and 145 in the multi-year model). The number of latent variables in each model was determined by the lowest PRESS. LOOCV was used to calculate Q^2 coefficients to assess the predictive power of the model. The predictor variables with the lowest standardized regression coefficients were removed by a forward selection process (Andersen and Bro 2010). This process was repeated

until only one predictor variable remained. The model selection was based on having the highest Q^2 value.

Results

Juice Chemistry. Table 2.1 contains the mean values of berry chemistry at harvest by year. Soluble solids (°Brix) and pH differed by season, with 2010 showing the highest accumulation of soluble solids ($p < 0.001$), highest pH ($p < 0.001$), and lowest TA ($p < 0.001$); 2011 the lowest concentration of soluble solids, and 2012, the lowest pH. Despite these differences in traditional indicators of ripeness, there were no significant differences in AMM ($p = 0.336$). The F-test of YAN by year appeared to be significant ($p = 0.033$), however the more conservative post hoc analysis with Tukey's method showed no significant differences in means, indicating a possible Type 1 error in the ANOVA F-test. PAN was significantly lower in 2012 ($p < 0.001$). An ANOVA comparing harvest YAN by vigor designations (data not shown) showed no significant differences between vigor designations ($p = 0.967$).

During the final two weeks of ripening, AMM concentrations decreased ($p < 0.001$) from a preharvest mean value of 56 mg/L to a mean harvest value of 45 mg/L. A statistically insignificant increase was observed in mean PAN concentration from 53 to 5 mg/L.

Table 2.1 Mean values and standard deviation of Riesling berry chemistry at harvest

		AMM (mg/L)		PAN (mg/L)		YAN (mg/L)		TA (g/L)		pH		Brix	
	n	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2012	50	45.8a[a]	25.2	42.3a	16.5	80.0a	34.9	8.2a	0.97	3.1a	0.07	18.8a	1.02
2011	40	48.8a	20.7	55.4b	19.4	99.8a	34.1	8.9a	0.75	3.2b	0.07	17.2b	1.11
2010	55	40.9a	31.1	63.7b	25.5	96.8a	47.1	7.2b	0.80	3.3c	0.10	19.9c	1.69
ANOVA		p=0.336		p<0.001		p=0.033		p<0.001		p<0.001		p<0.001	

[a]Within a column, means followed by a different letter are significantly different using Tukey's test. $p < 0.05$ was considered significant.

Probability Distribution. Figure 2.1A shows a histogram of YAN harvest data. The data fit a Gamma distribution skewed to the right. The low Anderson-Darling (AD) statistic (0.349) and high p -value (> 0.250) indicate a good fit to the distribution. The probability plot in Figure 2.1B shows the estimated population percentiles. The distribution predicts about 95% of the population will have a harvest YAN concentration between 30 and 190 mg/L; less than 1% will of samples contain more than 200 mg/L YAN at harvest.

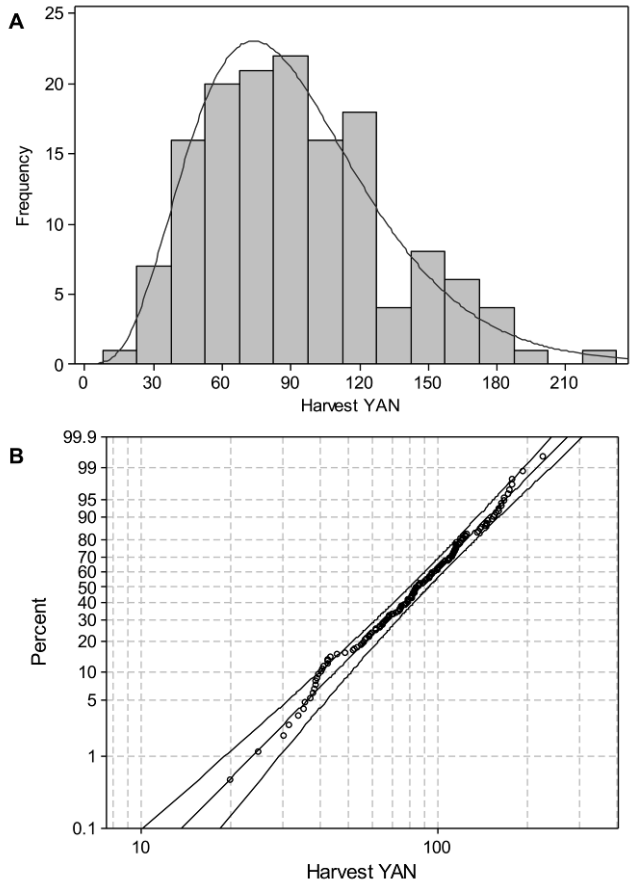

Figure 2.1 A histogram of harvest YAN data based on 145 observations from 3 years of data. B population percentiles based on gamma distribution with shape value of 4.996 and scale value of 18.42.

Yan Prediction Using Linear Regression. Linear regression models for individual years and combined data, significant at $p < 0.05$, successfully predicted YAN at harvest using preharvest measurements of YAN, AMM, and PAN. Table 2 summarizes the regression models for harvest YAN predictions using data collected two weeks prior to harvest, while Figures 2.2-

2.5 graphically represent the regression models. In models combining data from all three years, harvest YAN was best predicted by preharvest YAN measurements resulting in an R^2 of 71%, and LOOCV of the data resulted in Q^2 of 70%. Preharvest YAN was also the best predictor of harvest YAN in 2010 with a Q^2 of 75%.

Table 2.2 Linear regression model equations, correlation coefficients, and cross-validation for prediction of harvest YAN in Riesling grapes.

AMM[a]			
Year	Equation	R^2	Q^{2d}
2010	15.2 + 1.30 Preharvest AMM	77.20%	75.07%
2011	50.8 + 0.980 Preharvest AMM	59.60%	58.50%
2012	25.2 + 0.970 Preharvest AMM	68.90%	66.42%
All	30.6 + 1.08 Preharvest AMM	64.40%	63.26%
PAN[b]			
Year	Equation	R^2	Q^2
2010	9.70 + 1.42 Preharvest PAN	79.20%	77.09%
2011	60.6 + 0.723 Preharvest PAN	40.40%	28.16%
2012	34.8 + 1.16 Preharvest PAN	55.70%	52.11%
All	35.5 + 1.09 Preharvest PAN	61.30%	59.44%
YAN[c]			
Year	Equation	R^2	Q^2
2010	5.13 + 0.811 Preharvest YAN	84.80%	83.34%
2011	50.1 + 0.520 Preharvest YAN	55.10%	49.33%
2012	26.3 + 0.626 Preharvest YAN	66.80%	64.00%
All	25.9 + 0.669 Preharvest YAN	70.50%	69.51%
Previous Year YAN			
Year	Equation	R^2	Q^2
2010	-	-	-
2011	69.4 + 0.282 2010 Harvest YAN	16.30%	4.36%
2012	40.5 + 0.389 2011 Harvest YAN	19.00%	11.37%
All	57.4 + 0.303 YAN Previous Year	15.50%	11.35%

[a]AMM:ammonia; [b]PAN:primary amino nitrogen; [c]YAN: yeast assimilable nitrogen; [d]Q^2:Cross Validated R^{2h}

Preharvest ammonia concentrations provided the next best measure to predict harvest YAN concentrations. In a model using data from all three years it had a Q^2 of 63%. In 2011 and

2012, preharvest AMM was the best predictor of YAN at harvest with Q^2 of 59% and 66% respectively.

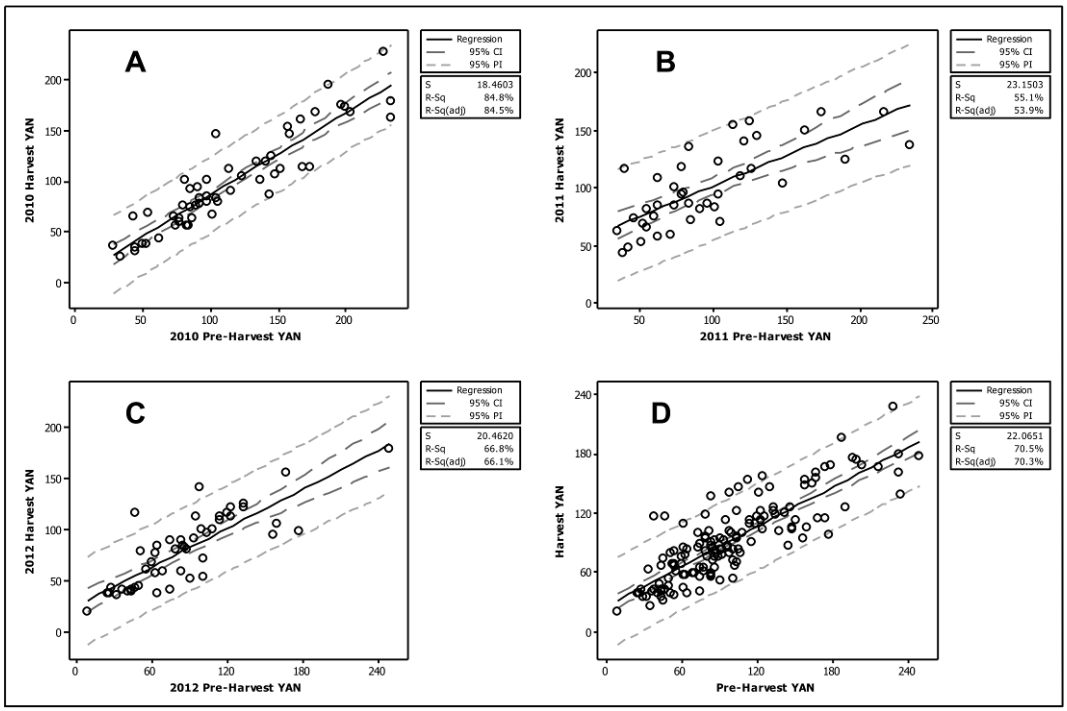

Figure 2.2 Fitted line plots of harvest YAN based on preharvest YAN measurements. Wide dashed lines indicate the 95% confidence interval where the mean value is likely to fall and the narrow dashed lines indicates the 95% prediction interval where individual values are likely to fall. A 2010 Preharvest YAN; B 2011 Preharvest YAN; C 2012 Preharvest YAN; D 2010-2012 Preharvest YAN

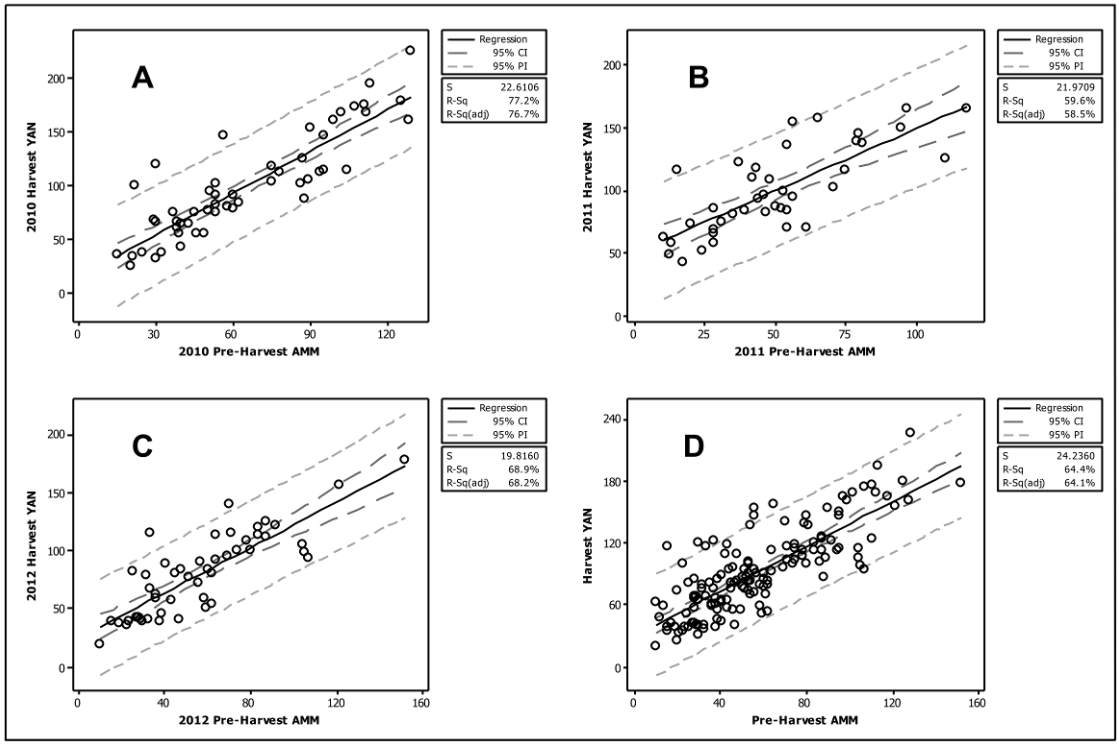

Figure 2.3 Fitted line plots of harvest YAN based on preharvest AMM measurements. Wide dashed lines indicate the 95% confidence interval where the mean value is likely to fall and the narrow dashed lines indicates the 95% prediction interval where individual values are likely to fall. A 2010 Preharvest AMM; B 2011 Preharvest AMM; C 2012 Preharvest AMM; D 2010-2012 Preharvest AMM

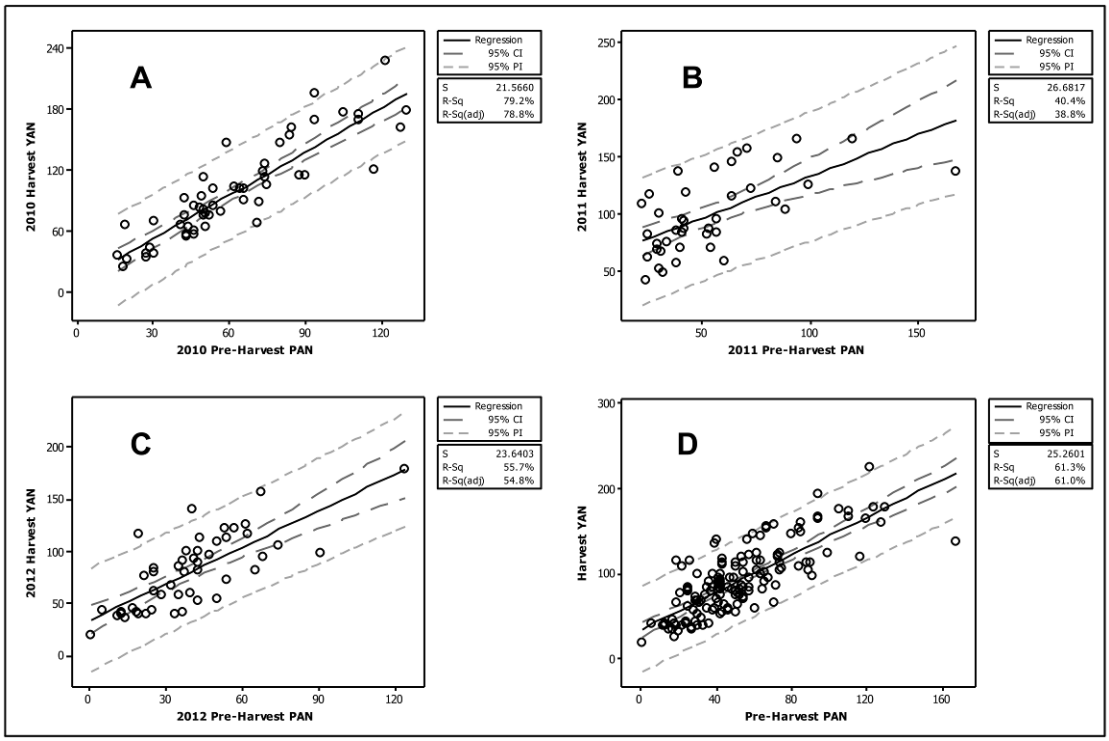

Figure 2.4 Fitted line plots of harvest YAN based on preharvest PAN measurements. Wide dashed lines indicate the 95% confidence interval where the mean value is likely to fall and the narrow dashed lines indicates the 95% prediction interval where individual values are likely to fall. A 2010 Preharvest PAN; B 2011 Preharvest PAN; C 2012 Preharvest PAN; D 2010-2012 Preharvest PAN

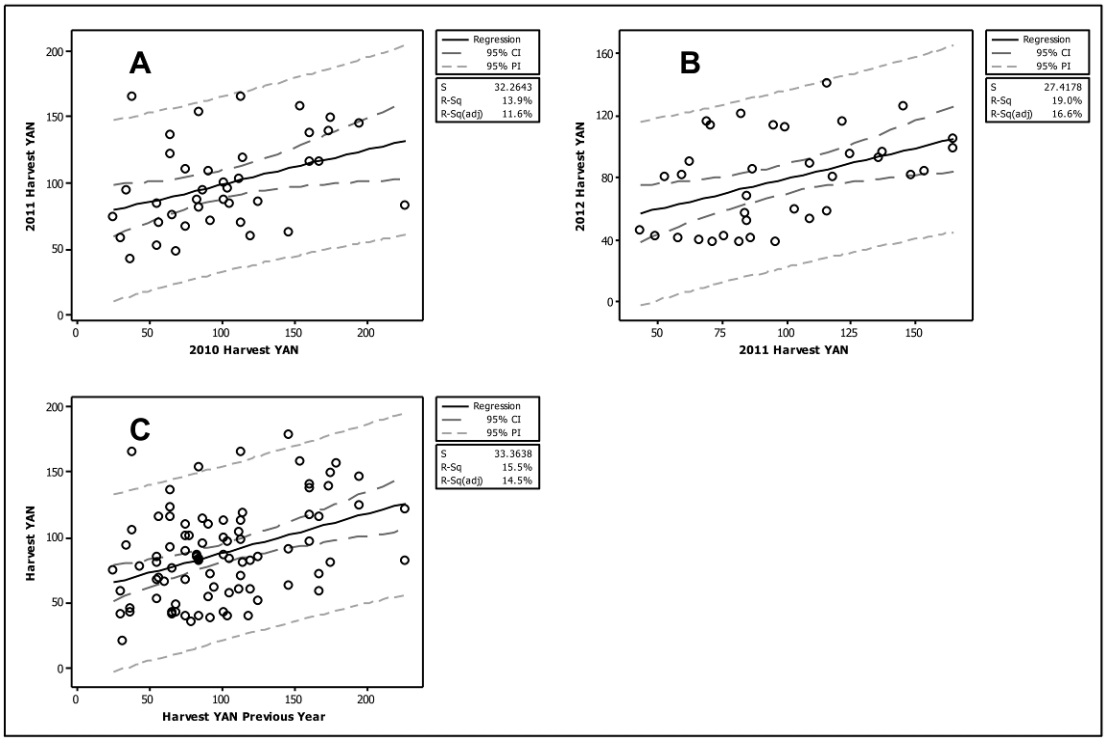

Figure 2.5 Fitted line plots of harvest YAN based on previous year harvest YAN measurements. Wide dashed lines indicate the 95% confidence interval where the mean value is likely to fall and the narrow dashed lines indicates the 95% prediction interval where individual values are likely to fall. A 2010 harvest YAN; B 2011 harvest YAN; C 2010&2011 Preharvest YAN

Preharvest PAN had the lowest R^2 and Q^2 of all preharvest nitrogen measures. For the individual year modes, 2010 had the best correlation between preharvest nitrogen measurements and harvest YAN, while 2011 had the lowest correlation.

Finally, harvest YAN data from the previous year explained the lowest amount of variation in observed responses, with an R^2 of 15% and a Q^2 of 11% for combined 2011 and 2012 data. Significant regression models ($p < 0.05$) were also achieved with YAN data collected at veraison to predict YAN at harvest (data not show). Despite this significance, the models had weak predictive power, with Q^2 values for models AMM, PAN, and YAN of 22%, 7%, and 18% respectively.

Yan Prediction using Multiple Linear Regression. Significant (p <0.05) MLR models could be constructed for harvest YAN (Table 2.3); R^2 and Q^2 represent the amount of variation

explained and the predictive power of the model, respectively. The MLR models in 2010 (R^2 = 85% Q^2 = 82%), 2012 (R^2 = 81% Q^2=76%), and all years (R^2 = 77% Q^2 = 74%) had higher R^2 and Q^2 values than the linear regression models. Only in 2011 did the MLR (R^2 = 61% Q^2 = 53%) have a slightly lower Q^2 values compared to the best linear regression (R^2 = 60%, Q^2 = 59%). None of the coefficients were used in more than two models. However, each model had either preharvest AMM or preharvest YAN as its most significant prediction variable. In the model combining data from all years, preharvest PAN concentration had a significant ($p < 0.05$) negative correlation with harvest YAN. Notably, no terms from veraison sampling were included in any of the models. Potassium content was the only soil component (collected in 2010) that was included in the regression model. Preharvest berry weight had marginal significance ($p = 0.096$) in 2010, and veraison berry weight was included in the 2011 model, but as discussed previously, the 2011 MLR model had low predictive power. No other prediction variables associated with berry weight, nor measures of Brix, were included in any of the models.

Table 2.3 Comparison of multiple linear regression models: model selection statistics, regression coefficients of x variables for the best model to predict YAN at harvest in Riesling berries at all sites in 2010 (N=49), 2011 (N=35) 2012 (N=48), and combined (N=132)

	Mode			
Coefficient	2010	2011	2012	All
Constant	69.14	2.935	-	-286.98
Soil K (mg/kg)	-	NA	NA	NA
T-Value	-1.93	NA	NA	NA
P-Value	0.063	NA	NA	NA
Veraison Berry	--	76	--	--
T-Value	--	2.03	--	--
P-Value	--	0.051	--	--
Veraison	--	--	--	0.098
T-Value	--	--	--	2.72
P-Value	--	--	--	0.007
Preharvest	-	--	--	--
T-Value	-	--	--	--
P-Value	0.096	--	--	--
Preharvest pH	--	--	-175	-74
T-Value	--	--	-2.69	-2.27
P-Value	--	--	0.010	0.025
Preharvest TA[b]	--	--	-12	--
T-Value	--	--	-3.24	--
P-Value	--	--	0.002	--
Preharvest	--	0.96	1.031	--
T-Value	--	7.03	11.8	--
P-Value	--	0.000	0.000	--
Preharvest	--	--	--	-0.46
T-Value	--	--	--	-1.99
P-Value	--	--	--	0.048
Preharvest	0.823	--	--	0.79
T-Value	13.88	--	--	6.56
P-Value	0.000	--	--	0.000
Harvest pH	--	--	308	153
T-Value	--	--	4.97	5.03
P-Value	--	--	0.000	0.000
Harvest TA[b]	--	--	18.7	7.4
T-Value	--	--	4.12	3.71
P-Value	--	--	0.000	0.000
S[e]	17.9	20.9	16	19.5
R^2	87.53	61.32	81.16	76.98%
R^2(adj)	86.32	58.90	78.91	75.88%
PRESS[f]	1210	1686	1369	54726.3
Q^{2g}	84.82	53.11	76.11	73.57%

-- indicates variable was not included in the model; NA: measure not available for that year; [a]AMM: ammonium; [b]TA: titratable acidity as tartaric acid equivalents; [c]PAN: primary amino nitrogen; [d]YAN: yeast assimilable nitrogen; [e]S: standard deviation of error; [f]PRESS: predicted error sum of squares; [g]Q^2: cross validated R^2

Multivariate Factor Analysis of Data. To determine how closely variables were related, factor analysis was conducted to visualize the covariance structure in the data. The

loadings for the first two factors suggest that measures of nitrogen preharvest and at harvest are strongly correlated along the first factor, while veraison measurements of nitrogen are correlated along the second. This multicolinearity indicates that some predictor variables are not independent, but rather are correlated with other predictors.

Table 2.4 Partial least squares regression models: basic statistics of the models and regression coefficients of x variables for the best model for YAN concentration at harvest in Riesling berries from the Finger Lakes in 2010 (N=49), 2011 (N=35) 2012 (N=48), and combined (N=132)

	Model			
Coefficient	2010	2011	2012	All
Constant	-205.52	48.125	-557.06	-141.58
K mg/Kg	-0.042	NA	NA	NA
Standardized	-0.097	NA	NA	NA
Preharvest pH	--	--	-137.870	-77.116
Standardized	--	--	-0.257	-0.225
Preharvest TA[a]	--	--	-13.495	-5.399
Standardized	--	--	-0.442	-0.144
Preharvest AMM[b]	0.398	0.514	0.984	0.502
Standardized	0.263	0.404	0.842	0.373
Preharvest PAN[c]	0.447	--	-0.457	--
Standardized	0.268	--	-0.295	--
Preharvest YAN[d]	0.256	0.273	0.236	0.360
Standardized	0.279	0.389	0.309	0.455
Harvest pH	49.197	--	312.012	117.354
Standardized	0.107	--	0.614	0.350
Harvest TA[a]	8.703	--	20.585	10.634
Standardized	0.148	--	0.572	0.253
NLV[e]	1	1	5	5
X Variance	0.549	0.9548	0.970	0.993
Error	12825.3	18114.4	10911.4	52101.6
R^2	86.18%	60.07%	81.62%	77.02%
PRESS[f]	15577.1	20143	14635.5	58558.1
Q^{2g}	84.43%	55.60%	75.34%	74.18%

-- indicates variable was not included in the model; NA: measure not available for that year; [a]TA: tiratable acidity as tartaric acid equivalents; [b]AMM: ammonia; [c]PAN: primary amino nitrogen; [d]YAN: yeast assimliable nitrogen; [e]NLV: number of latent variables; [f]PRESS: predicted error sum of squares; [g]Q^2: cross validated R^2

Partial Least Squares Regression. To compensate for suspected co-linearity of prediction variables, partial least squares regressions (PLSR) were constructed. Models ($p < 0.05$) with the highest Q^2 are shown in Table 2.4. The coefficients for each predictor were used to calculate the fitted value of the response variable, harvest YAN, while the standardized

coefficients give an indication of the relative importance of each predictor in the model. Harvest YAN was best predicted by either preharvest YAN or preharvest AMM. Notably, harvest pH and TA were included in 3 of the 4 models including the multi-year model. The number of latent variables in the models, inferred through principle components of predictor variables, ranged from 1 in 2010 and 2011 to 5 in the 2012 and the multi-year model. The response plot from the PLSR for all years in Figure 2.6 provides graphic representation of model prediction of harvest YAN.

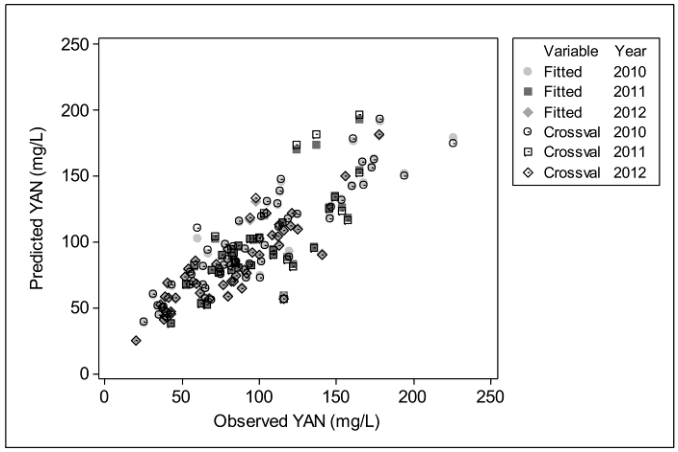

Figure 2.6 Plot of predicted vs. observed harvest YAN values calculated using the combined years partial least squares regression model. Each closed point represents an individual site, and different symbols represent different years. Open symbols represent the cross-validated value.

Discussion

Juice Chemistry. The differences observed in soluble solids, pH, and TA at harvest may be a result of the weather patterns during the growing seasons. 2010 and 2012 were similar growing seasons with warm springs leading to early bud break, approximately 3000 growing degree days, and timely rainfall (H. Walter-Peterson, Finger Lakes Vineyard Notes 2010, H. Walter-Peterson Finger Lakes Vineyard Notes, 2012). 2011 on the other hand was a cool wet spring with about average bud break, June – August was hot and dry, and September and October consisted of almost daily rainfall (H. Walter-Peterson, Finger Lakes Vineyard Notes 2011). The cooler temperatures in 2011 and heavy rainfall may account for the low level of soluble solids.

Population Distribution. In the population studied, juice samples from regional Riesling vineyards were generally found to be deficient in YAN, with average concentrations of 92.0 mg/L. Given the average deficiency, winemakers often supplement grape must prophylactically with additions of as much as the maximum legal U.S. addition of 200 mg N/L from DAP, an addition level which has been reported as common practice in the broader wine world (Ugliano et al. 2007). The probability distribution (Figure 1) for this population of samples predicts that 95% of sites will have a YAN concentration falling in the range between 29 mg/L and 190 mg/L, with a mean of 86 mg/L. Subsequently, an addition of 200 mg N/L would result in post addition YAN concentrations greater than 285 mg/L in most samples, with about 1% of samples having a YAN concentration greater than 400 mg/L. Winemakers in the Finger Lakes can use the population distribution data to make a better prophylactic addition of nitrogen. A lower dose of 120 mg N/L would ensure that less than 0.5% of samples from the population would have a concentration below 140 mg/L YAN, and less than 0.1% of samples would have a

concentration above 400 mg/L YAN. Further, the average concentration of samples would be 206 mg/L YAN, which is very close to the concentration recommended by Bisson and Butzke (2000) for musts at 21 Brix. In addition to the lower risk of excess nitrogen, lowering the prophylactic dose of nitrogen can reduce costs of nitrogen supplementation.

Linear regression. Of the models described, linear regression models using preharvest data provided the simplest method for harvest YAN prediction, requiring the least amount of data collection. Linear regression using preharvest YAN gave the best results in the multi-year model as well as in 2010, while preharvest AMM resulted in models with the best predictive power in 2011 and 2012 models (Table 2). Because AMM is one of two measurements required for assessing total YAN, the usefulness of preharvest AMM as a predictor of harvest YAN is of practical interest. Because AMM represents a single quantification, which can be performed using either spectrophotometric methods or an ion selective probe, it is less costly than total YAN measurement and provides more method flexibility.

Using the linear regression models, a 95% prediction interval for individual sites can be estimated. For example, the model predicts that at sites with preharvest YAN concentrations of 99 mg/L will have harvest YAN values that fall within a 95% prediction interval from 48.2 mg/L to 136.2 mg/L, a range of 88 mg/L. This is about half as wide as the range generated from the population distribution alone (160 mg/L). The smaller prediction interval allows winemakers to further reduce the amount of supplemented nitrogen without increasing the risk for nitrogen-deficient must.

Multiple Linear Regression. More complex models using MLR led to more accurate prediction models, as evidenced by the higher Q^2 values, compared favorably to the linear regression models. Despite better predictive power, the MLR models require more analysis to

obtain values for required predictor variables, and produced only incremental improvement in predictive power Q^2 = 74% compared to 70% for linear regression. In the models for individual years none of the measures were used more than once; however, each model did contain at least one measure of pre-harvest nitrogen. In the MLR model combining data from all three years, preharvest and harvest measurements of pH and TA were included, suggesting a correlation between these values and harvest YAN. Notably, pH and TA are both positively correlated to harvest YAN, but are inversely correlated to each other, which may imply buffering effects from YAN components. The fact that Brix measurements were not included in any of the MLR models is likely explained by Bell and Henschke (2005), who describe the conflicting changes that occur during ripening when AMM decreases, but PAN increases with increasing Brix. Similar trends were also observed in the Riesling ripening data (Table 2.1).

Partial Least Squares Regression. Factor analysis indicated multicolinearity between predictor variables, necessitating the use of PLSR techniques to select prediction variables from projections of latent variables. Like the MLR, the PLSR contains six coefficients, but selected based on the projections of five uncorrelated latent variables, effectively reducing correlation between predictor variables. The PLSR regression model had the highest predictive power of the three models used, with a Q^2 of 74%. Using the model requires additional analysis, compared with linear regression, to obtain values for the six predictor variables. The standardized coefficients, shown in Table 4, indicate the relative magnitude of the effect a predictor variable has on the model. In the PLS model Harvest YAN was best predicted by preharvest YAN measurements, followed by preharvest AMM, harvest pH, harvest TA, preharvest pH, and preharvest TA. pH and TA values at harvest remained important predictors, both positively correlated to harvest YAN despite being inversely correlated to each other.

While variation in amino acid accumulation and decreases in AMM make prediction models cultivar-dependent, it is notable that an efficient predictive model could be developed from data representing a range of sites and climatic variation. These data suggest that regression models can be made to predict harvest YAN in Finger Lakes Riesling using measurements taken two weeks prior to harvest. Winemakers could employ simple linear equations adapted from the regression models to obtain an estimate of harvest YAN concentrations.

Application of the Models. Equations from the models (tables 2, 3, and 4) can be applied to predict new observations in the population (i.e. Finger Lakes Riesling sites) within a prediction interval (PI). Harvest YAN values were calculated for new observations using mean values of predictor variables. The predicted value and the 95% PI are shown.

Linear Regression.

Harvest YAN = 1.08[Preharvest AMM] + 30.6

= 1.08[56.27 mg/L] + 30.6

= 91.16 ±48.08 mg/L

Harvest YAN = 0.669[Preharvest YAN] + 25.9

= 0.669[99.23 mg/L] + 25.9

= 92.31 ±43.78 mg/L

Multiple Linear Regression.

Harvest YAN = 0.098[Veraison AMM] -74[Preharvest pH] – 0.46[Preharvest PAN] + 0.79[Preharvest YAN] + 152.54[Harvest pH] + 7.4[Harvest TA] -286.98

= 0.098[115.45 mg AMM/L] -74[3.12] – 0.46[52.87 mg PAN/L] + 0.79[99.23 mg YAN/L] + 152.54[3.16] + 7.4[8.24 g/L] -286.98

= 91.18 ±38.8

Partial Least Square Regression.

Harvest YAN = -77.12[Preharvest pH] – 5.40[Preharvest TA] + 0.50[Preharvest AMM] + 0.36*[Preharvest YAN] + 117.35[Harvest pH] + 10.63[Harvest TA] - 141.58

= -77.12[3.12] – 5.40[9.05 g/L] + 0.50[56.27 mg AMM/L] + 0.36*[99.23 mg YAN/L] + 117.35[3.16] + 10.63[8.24] - 141.58

= 91.43 ±39.31

The 95% PI gives the range where new observations are likely to fall, the models with better predictive power result in a smaller PI. Using this information winemakers can decide whether the improvement in the PI justifies the additional analysis required to obtain values for the prediction variables. In many cases measuring AMM only two weeks before harvest may provide enough accuracy to calculate successful nitrogen additions.

Conclusions

It is well understood that nitrogen concentrations can affect fermentation parameters, but the difficulty of measurement and long lead times for external analysis cause many winemakers to forgo analysis and rely on prophylactic additions for healthy fermentations. Using probability distributions based on harvest nitrogen concentrations collected from 62 commercial Riesling vineyards over three years, better estimates of appropriate prophylactic additions to minimize the risk of nitrogen deficiency or excess in Riesling must can be made. Statistically significant linear regression models were developed that further reduce the prediction interval for vineyard sites in the Finger Lakes. In Riesling, preharvest YAN gives the best prediction of harvest YAN, however, preharvest AMM values predict almost as well, and may be easier to measure. Finally, more complex MLR and PLSR models result in better predictive power, although they may not

be practical because they require additional calculation and measurements. The successful development of prediction models for harvest YAN in Riesling grapes from the Finger Lakes region suggests that this method may be used to develop similar models for specific cultivars and growing regions.

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CHAPTER 3

ACCUMULATION AND PREDICTION OF YEAST ASSIMILABLE NITROGEN IN NEW YORK WINE GRAPE CULTIVARS

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Abstract: A three-year study was conducted to investigate the accumulation of yeast assimilable nitrogen (YAN) during the final weeks of ripening in seven wine grape cultivars grown in New York State, and to assess the feasibility of predicting harvest YAN using linear regression models. Berry samples of Cabernet Franc, Chardonnay, Merlot, Noiret, Pinot noir, Riesling, and Traminette were collected weekly from 49 vineyard sites across the Finger Lakes, Hudson Valley, Lake Erie, and Long Island growing regions from August through harvest. YAN concentrations in Cabernet Franc, Riesling, and Traminette were generally low, averaging below 100 mg/L annually, while Chardonnay and Pinot noir showed average YAN concentrations greater than 200 mg/L. During the ripening period, linear regression models were found to predict harvest YAN up to five weeks prior (R^2=81.6%). A decrease in YAN during ripening was observed across cultivars, caused primarily by decreases in ammonia (AMM), as PAN levels remained stable. Population distributions were used to estimate appropriate prophylactic nitrogen additions for each cultivar, minimizing the risk of deficiency or excess; this was most difficult with Chardonnay, Noiret, and Pinot noir, which had the highest and most variable YAN concentrations, and subsequently run the greatest risk of over-supplementation with prophylactic additions.

Introduction

The concentration of yeast assimilable nitrogen (YAN) in grapes at harvest is highly variable, and differences exist between cultivars (Butzke 1998, Hagen et al. 2008). Some cultivars, such as Riesling, often exhibit low YAN concentrations (Stines et al. 2000, Nisbet et al. 2013), which can lead to stuck or sluggish fermentations and subsequent production of 'reduced' sulfur aromas (Ugliano et al. 2009, Acree et al. 1972, Vilanova et al. 2007, Vos and Gray 1979). To prevent such problems, winemakers often supplement grape must with inorganic nitrogen in the form of diammonium phosphate (DAP), or with more complex nutrient blends containing both inorganic ammonia (AMM) and primary amino nitrogen (PAN). While still subject to debate, it is generally accepted that a YAN concentration of 140 mg/L is the minimum required for healthy fermentation (Bely et al. 1990), and that optimum concentration is related to percent soluble solids (Bisson and Butzke 2000). In high nitrogen musts, yeast have been found to consume a maximum of 400 mg/L YAN during fermentation (Bisson and Butzke 2000); additions beyond that concentration may result in residual nitrogen post-fermentation, subsequent microbial instability, and spoilage (Bell and Henschke 2005). Residual nitrogen may also be used by lactic acid bacteria, which produce biogenic amines through decarboxylation of amino acids (Moreno-Arribas et al. 2003), and can lead to the formation of ethyl carbamate, a known carcinogen which causes headache and respiratory distress in susceptible individuals (Monteiro et al. 1989, Bach et al. 2011, Daudt et al. 1992, Jansen et al. 2003).

Given the importance of optimizing YAN in must, appropriate estimation of initial must nitrogen is an important step in determining appropriate YAN additions. There are many methods for nitrogen determination in grape must, with various advantages and limitations, but all require significant laboratory equipment and analytical skill (Gump et al. 2002). The time

and expertise required often prevents small and medium-sized wineries from performing YAN analysis. Time can also be a limiting factor if samples are submitted to external service laboratories, as results may not arrive rapidly enough to make useful supplementation decisions. For these reasons, prophylactic nitrogen additions are common, and may frequently lead to YAN concentrations outside the optimum range.

In a previous study, regression techniques were used to predict harvest YAN concentration in Finger Lakes Riesling using pre-harvest measurements (Nisbet et al. 2013), but the use of only one grape cultivar and three sample points limited data resolution. Because changes in YAN may be cultivar dependent, developing regression models for different cultivars and species is important for early prediction of YAN. The amino acid concentration has been shown to increase during grape berry ripening, but can plateau or decrease prior to harvest depending on the cultivar (Hilbert et al. 2003, Hernández-Orte et al. 1999). Post-veraison increases in amino acid concentration are caused largely by proline accumulation, which can also vary by cultivar (Stines et al. 2000). Conversely, AMM has been shown to decrease in many cultivars throughout ripening (Bell and Henschke 2005).

The objective of this study is to develop cultivar-specific regression models for seven wine grapes grown across New York State, using measures of Brix, pH, TA, AMM and PAN collected weekly from August through October. In addition to developing predictive models for each cultivar, this work further elucidates changes in nitrogen content in relationship to other traditional parameters of ripeness, and allows comparison among cultivars.

Materials and Methods

Experimental Design. This survey, conducted over three harvests (2010-2012), examined the accumulation of YAN during the final stage of berry development in seven grape

cultivars: Cabernet Franc, Chardonnay, Merlot, Noiret, Pinot noir, Riesling, and Traminette. Cultivars were sampled at forty-nine commercial vineyard sites from four growing regions in New York State: Finger Lakes, Hudson Valley, Lake Erie, and Long Island (Table 1). The sampling area at each site was defined as twelve vines per row in two adjacent rows, for a sample unit of 24 vines. Sample sites were selected, with input from vineyard managers and extension associates across New York State, to capture a range of climate, soil types, and vineyard management practices. Vine management and harvest decisions were made by vineyard managers according to their own best practice methods.

Sample Collection and Processing. Grape berry samples were collected beginning 23 August in 2010, 29 August in 2011, and 27 August in 2012, and sampling continued weekly until grapes were harvested. Samples were collected for four to eight weeks depending on harvest date, which was determined by individual vineyard managers for each cultivar and site. The last available sample at a site each year was recorded as the harvest sample. Samples of one hundred berries were weighed on an Ohaus Pioneer PA3102 scale (Ohaus Corp. Pine Bluff, NJ), accurate to 0.01 g, to obtain fresh berry weight. Berries were then crushed immediately using a Stomacher® 400 paddle blender (Seward Laboratory Systems, Port Saint Lucie, FL) at 120 RPM for 60 seconds. Post stomaching, 50 mL of must was decanted from macerated berries for analysis.

Table 3.6: Vineyard sources of grape berry samples, 2010-2012.

Cultivar	Region	2010	2011	2012
Cabernet Franc	Finger Lakes	X	X	X
	Finger Lakes	X		X
	Finger Lakes	X	X	X
	Finger Lakes	X		
	Finger Lakes	X		
	Hudson Valley	X	X	X
	Lake Erie	X	X	X
	Long Island	X		X
	Long Island			X
Chardonnay	Finger Lakes	X	X	
	Finger Lakes	X		
	Finger Lakes	X	X	X
	Finger Lakes			X
	Hudson Valley	X	X	X
	Hudson Valley		X	
	Hudson Valley			X
	Long Island	X	X	X
	Long Island	X		
Merlot	Hudson Valley	X	X	X
	Long Island	X	X	X
	Long Island	X		X
Noiret	Finger Lakes	X		
	Finger Lakes	X	X	X
	Hudson Valley	X	X	X
	Hudson Valley	X	X	X
	Lake Erie		X	X
	Lake Erie	X		
Pinot noir	Finger Lakes	X	X	X
	Hudson Valley	X	X	X
	Hudson Valley	X	X	
	Hudson Valley			X
Riesling	Finger Lakes	X	X	X
	Finger Lakes	X		
	Finger Lakes		X	X
	Finger Lakes	X	X	X
	Finger Lakes	X	X	
	Finger Lakes	X	X	X
	Finger Lakes	X	X	X
	Finger Lakes	X		
	Finger Lakes			X
	Finger Lakes			X
	Hudson Valley	X	X	X
	Lake Erie	X	X	X
	Long Island	X	X	X
Traminette	Finger Lakes	X		
	Finger Lakes	X	X	X
	Finger Lakes		X	
	Hudson Valley	X	X	X
	Hudson Valley	X	X	X
	Lake Erie	X	X	X

Juice Chemistry. A 2mL aliquot was drawn from the 50mL juice sample, placed in a microfuge tube and centrifuged at 12000 x *g* in an Effendorf 5415C (Brinkmann Instruments,

Westbury, NY) for 2 min prior to analysis for YAN (AMM + PAN). A Chemwell 2910 multianalyzer (Unitech Scientific, Hawaiian Gardens, CA) was used to rapidly test samples. AMM was determined by the glutamate dehydrogenase (GDH) catalyzed condensation of ammonia and alpha ketoglutarate (ak-G) and simultaneous oxidation of nicotinamide adenine dinucleotide (NADH) (Ough 1969). The oxidation of NADH results in a decrease in absorbance at 340nm, which can be quantified by spectrophotometry (Unitech Scientific, Ammonia Extended range UniTAB 2007). PAN is determined by derivitization of primary amino groups by o-phthaldialdehyde and N-acetyl-l-cysteine (OPA/NAC) to form isoindoles, which are detected spectrophotometrically at 340 nm (Dukes and Butzke 1998) (Unitech Scientific, Primary Amino Nitrogen UniTAB, 2007).

Soluble solids (Brix) were measured using a digital refractometer (model 30016, Sper Scientific, Scottsdale, AZ) with temperature correction. Titratable acidity was measured with an auto-titrator (Titrino 798, Metrohm, Riverview, FL) and expressed as tartaric acid equivalents. pH was measured with an Accumet Excel XL 25 pH meter and an ion selective probe (Fisher Scientific, Waltham, MA).

Statistical Analysis . Data analysis was performed using Minitab 16 (Minitab, Reading, MA) statistical analysis software. Multiple analysis of variance (MANOVA) was used to simultaneously test the equality of means in the unbalanced design from the seven measures of fruit chemistry: Berry wt., Brix, pH, TA, AMM, PAN, and YAN. A one-way analysis of variance (ANOVA) was used to assess differences in berry chemistry values by cultivar at harvest. Tukey's method was used post hoc to separate means, and a p-value less than 5% was considered significance for each test.

Probability plots were used to evaluate the fit of a distribution of the YAN at harvest for each cultivar and estimate percentiles. Suitable distributions were selected by assessing fit using the lowest Anderson-Darling (AD) statistic, and a p-value > 0.05 to indicate that the population did not differ significantly from the distribution.

Results

Juice Chemistry. Grape cultivars exhibited differences in berry chemistry at harvest (Table 3.2). Riesling and Cabernet Franc had lower mean YAN concentration than Pinot noir, Chardonnay, and Noiret. Mean concentrations in Cabernet Franc, Riesling, and Traminette were consistently lower than the 140 mg/L minimum, while Chardonnay and Pinot noir concentrations were consistently higher (Figure 3.1). Merlot and Noiret varied by year. Riesling had one of the lowest mean concentrations of total YAN, but the third highest mean concentration of AMM, and the highest percentage (49%) of total YAN present as AMM. Riesling also had the lowest Brix at harvest and the highest TA. Pinot noir and Chardonnay were not different from each other in any of the categories measured. Pearson correlation coefficients and p-values for the response values suggest that YAN and PAN did not correlate to the time variable, i.e., these parameters did not change over time. AMM and TA decreased as harvest approached, while pH and Brix increased (Table 3.3). The correlation coefficients are confirmed in plots of mean nitrogen concentrations, where AMM shows a clear decline in all cultivars over time and PAN does not change (Figure 3.2). Changes in Brix, pH, TA and berry weight by cultivar were as expected, with Brix, pH, and berry weight increasing and TA decreasing during the ripening phase (Figure 3.3).

Table 3.7: Harvest chemistry of berry samples for seven NY wine grape cultivars.

Cultivar	n	YAN[a] (mg N/L) Mean±SD	AMM[b] (mg N/L) Mean±SD	PAN[c] (mg N/L) Mean±SD	Brix Mean±SD	pH Mean±SD	TA[d] (g/L TAE) Mean±SD	Berry wt (g) Mean±SD
Cabernet Franc	19	75±44d[e]	14±14d	64±35cd	21.6±1.4a	3.47±0.23b	6.3±1.3cd	1.6±0.2abc
Chardonnay	16	200±83ab	64±30a	148±61a	20.7±1.5ab	3.53±0.23b	7.1±1.2abc	1.5±0.2c
Merlot	8	132±47bcd	31±15bcd	107±37abc	20.3±1.1abc	3.79±0.15a	4.9±0.6d	1.8±0.3a
Noiret	11	164±73abc	26±18cd	143±59ab	19.3±1.4bc	3.45±0.17b	7.2±1.1abc	1.7±0.3ab
Pinot noir	9	220±89a	63±23ab	169±78a	21.4±2.0ab	3.65±0.20ab	6.6±1.0bcd	1.4±0.3c
Riesling	29	84±52d	42±30bc	50±29d	18.5±1.6c	3.17±0.17c	8.1±1.0a	1.6±0.1bc
Traminette	14	95±25cd	8±4d	89±25bcd	20.7±1.9ab	3.18±0.18c	7.9±1.7ab	1.7±0.2ab

[a]YAN: yeast assimilable nitrogen
[b]AMM: ammonia
[c]PAN: primary amino nitrogen
[d]TA: titratable acidity expressed in tartaric acid equivalents (TAE)
[e]Within a column, different letters are indicate significance at $p < 0.05$ (Tukey's test.)

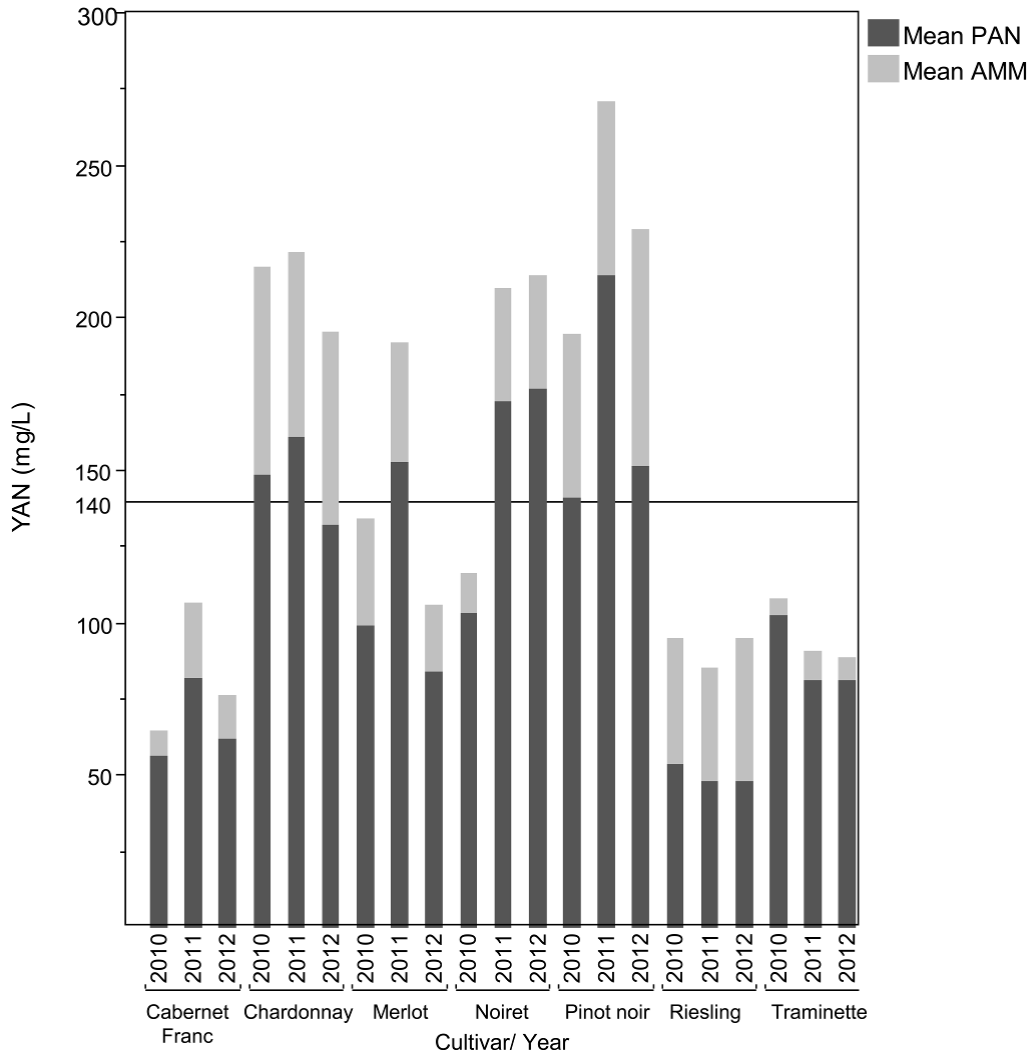

Figure 3.5: Mean concentration of AMM and PAN at harvest for seven grape cultivars, 2010-2012.

Table 3.8: Correlation coefficients across seven wine grape cultivar parameters during ripening.

		Weeks Preharvest	AMM[a]	PAN[b]	YAN[c]	Brix	pH	TA[d]
AMM[a]	r	-0.18						
	p-value	<0.001						
PAN[b]	r	0.057	0.514					
	p-value	0.155	<0.001					
YAN[c]	r	-0.02	0.754	0.95				
	p-value	0.62	<0.001	<0.001				
Brix	r	0.597	-0.315	0.045	-0.08			
	p-value	<0.001	<0.001	0.26	0.047			
pH	r	0.245	0.082	0.472	0.389	0.45		
	p-value	<0.001	0.041	<0.001	<0.001	<0.001		
TA[d]	r	-0.542	0.207	-0.175	-0.056	-0.745	-0.649	
	p-value	<0.001	<0.001	<0.001	0.19	<0.001	<0.001	
Berry Weight[e]	r	-0.031	-0.056	-0.078	-0.08	-0.039	-0.048	-0.037
	p-value	0.434	0.166	0.054	0.049	0.33	0.232	0.387

[a]AMM: ammonium
[b]PAN: primary amino nitrogen
[c]YAN: yeast assimilable nitrogen
[d]TA: titratable acidity as tartaric acid equivalents

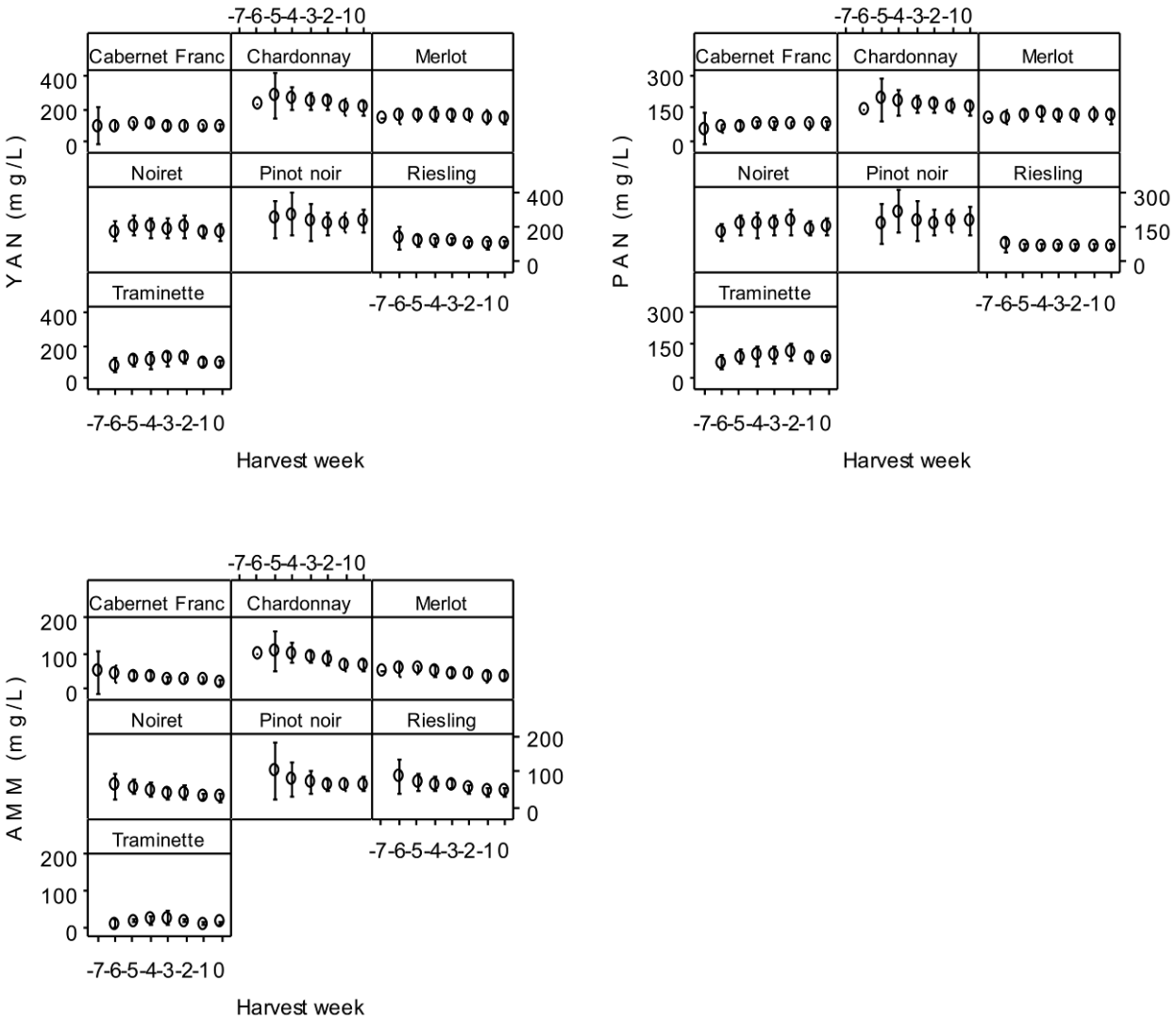

Figure 3.6: Interval plot depicts changes (A) YAN: yeast assimilable nitrogen (mg/L), (B) PAN: primary amino nitrogen (mg/L), and (C) AMM: ammonia (mg/L) in grape cultivars over time. Error bars represent 95% confidence intervals for the mean.

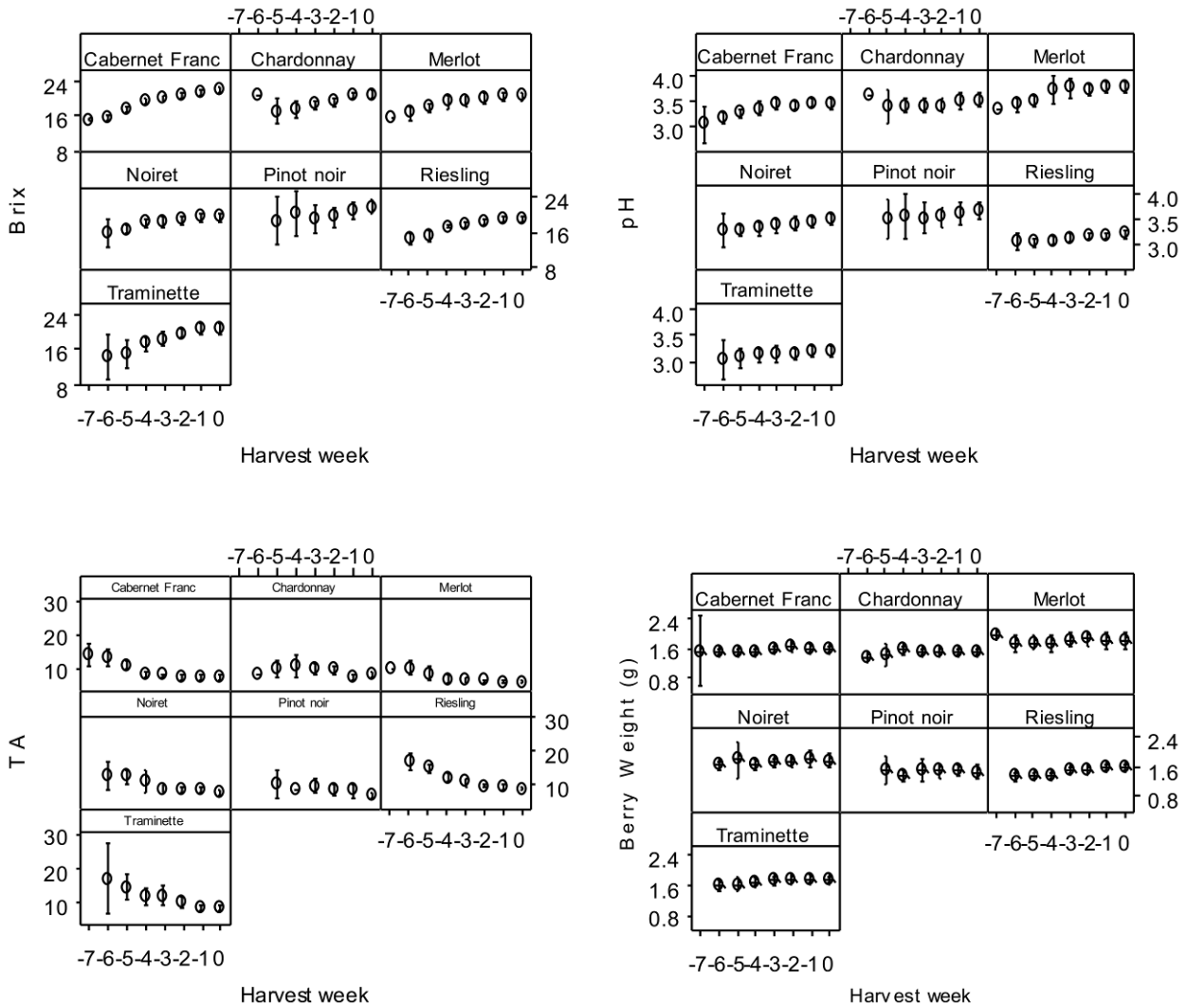

Figure 3.3: Interval plot depicts changes in (A) Brix, (B) pH, (C) TA in titratable tartaric acid equivalents and (D) berry weight in grape cultivars over time. Error bars represent 95% confidence intervals for the mean.

Population distribution. To address differences in sample populations and YAN values at harvest, probability plots were created for each cultivar to determine normal distribution and estimate population percentiles (Figure 3.4). The distribution of sample points showed normal distribution for all cultivars except Riesling (p=0.044), which was significantly different from the normal distribution, fitting a gamma distribution instead.

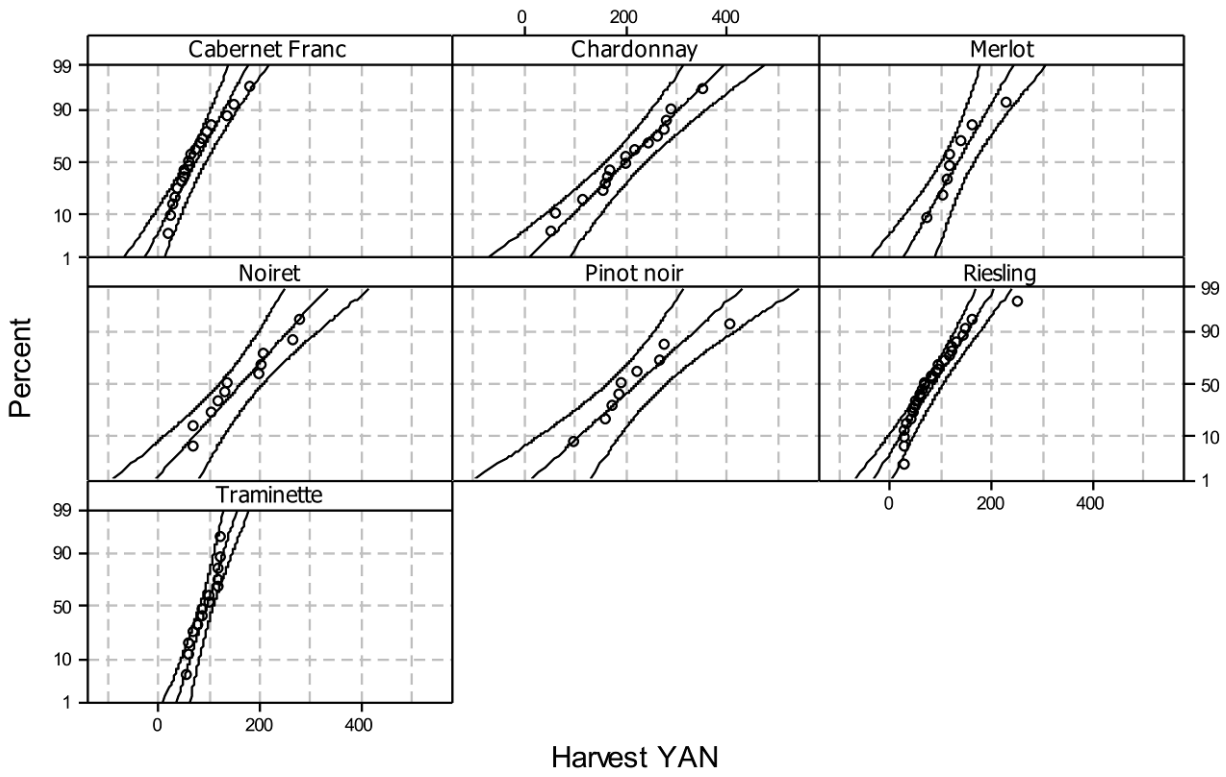

Figure 3.7: Population percentiles based on normal distribution of YAN concentration for each cultivar.

Using these distributions, likely YAN concentration ranges were calculated for each cultivar. Traminette had the smallest range, with YAN values likely to fall between 45 mg/L and 145 mg/L, and 96% likely to be below the 140 mg/L minimum needed for fermentation health. Pinot noir had the widest range (46 mg/L to 394 mg/L) followed closely by Chardonnay (38 mg/L to 362 mg/L). Cabernet Franc, Riesling, and Traminette each have a greater than 90% chance of having YAN concentrations below 140 mg/L, while Chardonnay, Pinot noir, and Noiret had a much lower risk of YAN deficiency (25%, 18% and 33% respectively).

Linear Regression. A linear regression was created for each cultivar to assess correlation between harvest and pre-harvest YAN concentrations (Figure 5). Cabernet Franc, Chardonnay, and Merlot showed the best fit between harvest YAN and measurements taken two weeks prior to harvest, with R^2 of 89.5%, 89.9%, and 95.5% respectively. Pinot noir

(R^2=54.1%) and Traminette (R^2=64.5%) showed poor correlation, and Riesling a moderate correlation (R^2=78.0%). Linear regressions of all cultivars using YAN concentrations at 2 weeks, 3 weeks, 4 weeks, and 5 weeks before harvest all showed R^2 greater than 80% (Figure 6).

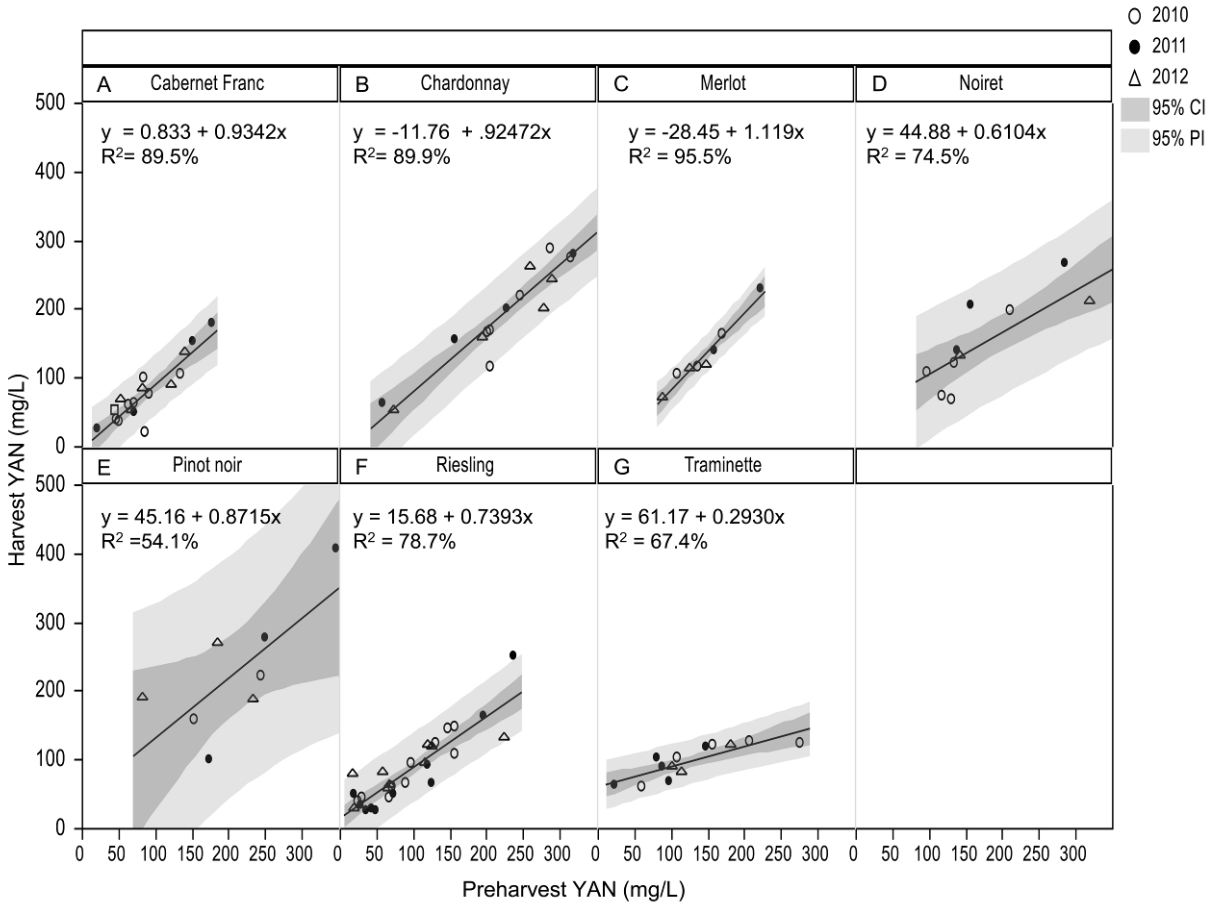

Figure 3.8: Fitted line plots of harvest YAN based on preharvest YAN measurements for grape cultivars. Dark shaded area indicate the 95% confidence interval where the mean value is likely to fall, and the lighter shading indicates the 95% prediction interval where individual values are likely to fall. A = Cabernet Franc, B = Chardonnay, C = Merlot, D = Noiret, E = Pinot noir, F = Riesling, G = Traminette.

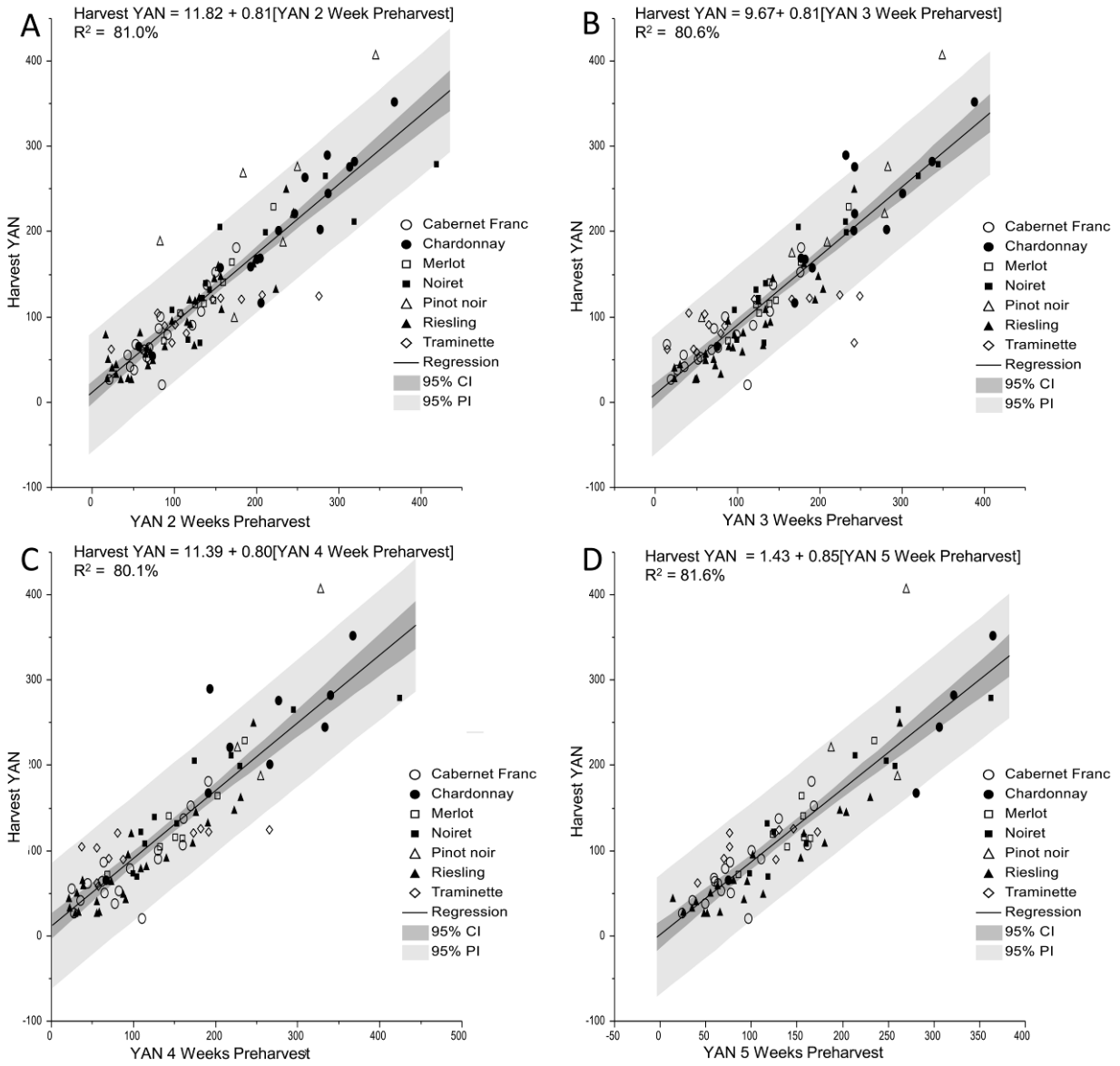

Figure 3.9: Linear Regression of harvest YAN by preharvest measurements for grape cultivars. The dark shaded area represents the 95% confidence interval where the mean value is likely to fall, and the light shaded area is the 95% prediction interval where individual values are likely to fall. A = 2 weeks preharvest, B = 3 weeks preharvest, C = 4 weeks preharvest, D = 5 weeks preharvest.

Analysis of Variance. A MANOVA was used to simultaneously compare the seven response variables using cultivar, weeks preharvest, year, and the interaction terms 'cultivar x year' and 'cultivar x week preharvest' as factors. Wilks' lambda indicated significant model effects for both factors and the interaction term for at least one response variable (Table 4), showing that responses vary by cultivar from year to year ('cultivar x year') and during the

preharvest sampling interval ('cultivar x week preharvest'). The univariate ANOVA suggested that cultivar differences existed for each response factor with $p<0.001$ (Table 4). Not all responses, however, showed changes over time; notably, PAN had a p value >0.05. The interaction term 'cultivar x year' showed that for YAN, PAN, and AMM cultivar effects were different from year to year. Finally, Brix and TA both have $p<0.001$ for the interaction term 'cultivar x weeks preharvest,' indicating that the accumulation of soluble solids and decrease in acidity over the preharvest sampling interval differ by cultivar.

Table 3.9: Effects of cultivar, week before harvest, harvest year and interactions on grape chemistry attributes using multiple analysis of variance (MANOVA); $\alpha = 0.05$ was used to determine significance.

	MANOVA	ANOVA						
Effects	Wilks' Lambda	YAN[a]	PAN[b]	AMM[c]	Berry Wt	Brix	pH	TA[d]
Cultivar	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Week Preharvest	0.002	0.045	0.652	< 0.001	0.002	< 0.001	< 0.001	< 0.001
Year	< 0.001	< 0.001	< 0.001	0.229	0.003	< 0.001	< 0.001	0.056
Cultivar*Year	< 0.001	< 0.001	< 0.001	< 0.001	0.704	0.092	0.345	0.452
Cultivar*Week Preharvest	< 0.001	0.630	0.917	0.150	0.087	< 0.001	0.742	< 0.001

[a]YAN: yeast assimilable nitrogen
[b]PAN: primary amino nitrogen
[c]AMM: ammonia
[d]TA: titratable acidity as tartaric acid equivalents

Discussion

Changes in AMM, PAN, and YAN during ripening. Unlike Brix and TA, which showed respective increase and decrease during ripening, YAN concentration was notable in its relative stability over time (Table 3.3). Overall, mean YAN concentration across cultivars decreased by only 15 mg/L, from 140 to 125 mg/L, during the final month of ripening. This decrease in YAN corresponds to the decrease in mean AMM from 50 to 35 mg/L, while mean PAN concentration remained stable. This runs contrary to previous data describing PAN as

increasing from veraison to harvest, and in some instances peaking prior to harvest (Bell and Henschke 2005). One explanation may be accumulation of proline; Stines et al (2000) showed that while proline increases steadily during ripening, arginine, the largest source of PAN, remains constant. Since PAN makes up more than 70% of YAN, the strong correlation found between the two ($r = 0.95$) is not surprising (Table 3). The decrease in AMM is likely responsible for the inverse correlation of both AMM and YAN to Brix (Table 3), though the correlation between YAN and AMM, at $r = 0.754$, is relatively weak (Table 3). AMM accounts for about 26% of YAN, so even a large percentage decrease in AMM during ripening has smaller, but significant impact on total YAN. One exception to this might be Riesling, which has about 48% of YAN present as AMM, the highest proportion among the cultivars studied.

In a previous study of Riesling grapes (Nisbet et al. 2013), it was found that regressions created from YAN measurements taken at veraison (approximately 8 weeks preharvest) correlated poorly with harvest samples ($Q^2 = 18\%$), but those taken two weeks preharvest were useful for predicting harvest YAN ($Q^2 = 70\%$). Subsequently, it was hypothesized that correlation to harvest YAN would decrease with earlier sampling dates; instead, the R^2 remained unchanged for regressions calculated from two to five weeks preharvest,. It appears, therefore, that PAN plateaus and stabilizes, and AMM begins to decrease, at a point shortly after veraison. After this point, decreases in YAN are predictable, and correlations with harvest YAN are strong.

Cultivar differences. The MANOVA and subsequent ANOVAs show that the rate of change in Brix and TA, the traditional measures of ripening, differs by cultivar (Table 4). Similarly, YAN concentration varied by cultivar, as previously reported (Butzke 1998). In this work, AMM decreased as berries ripened, but the rate of decrease was not cultivar dependent

(Table 4). No significant changes in PAN were observed in any of the cultivars studied. This further demonstrates that consistent differences in YAN concentration exist between cultivars (Table 2, Table 4). Chronic nitrogen deficiency was observed in Cabernet Franc, Riesling, and Traminette; conversely Chardonnay and Pinot noir showed averages consistently above 140 mg/L (Figure 1). The significance of the interaction between cultivar and year in the MANOVA (table 4) indicates that cultivars react differently to yearly climactic changes. Further, factors associated with growing season may have a greater effect on YAN components than they do on traditional measures of ripeness Brix, pH, and TA. This reinforces the observation made by Nisbet et al (2013) that YAN data from previous years is not a reliable predictor of future YAN values. Regional microclimate may enhance YAN deficiency in some cultivars. Stewart (2013) reported that NY had among the lowest average YAN concentrations compared to other wine growing regions in the United States, finding that Cabernet Franc, Riesling, and Traminette were all lower in New York than other regions, while Chardonnay showed slightly higher average YAN concentration than the average of all regions combined.

The strong correlation between PAN and YAN observed in this work is contrary to that reported previously in Riesling, where a stronger correlation was found between AMM and YAN (Nisbet et al. 2013). This is likely because Riesling has the highest ratio of AMM/YAN of the cultivars studied, with AMM accounting for more than 48% of YAN, compared to an average of 22% for other cultivars (as calculated from Table 2).

Linear Regression per cultivar. As was observed previously, YAN concentration two weeks pre-harvest can be used to predict harvest YAN in Finger Lakes Riesling (Nisbet et al. 2013). In this work, similar predictions could be made for many cultivars. The regression equation can be used to predict new observations from samples taken two weeks preharvest, and

the root mean square error (RMSE) term can be used to construct a 95% prediction interval, or approximately ±2 (RMSE). The regression equations and prediction intervals for each cultivar are as follows:

Cabernet Franc Harvest YAN = 0.833 + 0.9342 [Preharvest YAN] ± 28.6

Chardonnay Harvest YAN = -11.76 + 0.9247 [Preharvest YAN] ± 54.3

Merlot Harvest YAN = -28.45 + 1.119 [Preharvest YAN] ± 21.4

Noiret Harvest YAN = 44.88 + 0.6104 [Preharvest YAN] ± 77.2

Pinot noir Harvest YAN = 45.16 + 0.8715 [Preharvest YAN] ± 136

Riesling Harvest YAN = 15.68 + 0.7393 [Preharvest YAN] ± 48.32

Traminette Harvest YAN = 61.17 + 0.2930 [Preharvest YAN] ± 28.6

Noiret and Pinot noir showed large residuals from the regression line, resulting in large error terms and weaker predictions relative to other cultivars.

Nitrogen addition recommendations by cultivar. Population distributions (Figure 4) can be used to determine appropriate prophylactic nitrogen additions for each cultivar, as well as the percentage of grape lots predicted by the probability plot to be deficient (YAN < 140 mg/L) or in excess (YAN >400 mg/L) (Table 3.5). When YAN measurements are unavailable, this data can be used to estimate addition rate with little risk of excessive addition. Chardonnay, Pinot noir, and Noiret have the largest error and average YAN concentrations closest to the optimum value of 200mg/L, making prediction more difficult. Subsequently, prophylactic nitrogen supplementation for these cultivars runs a higher risk of over- or under-supplementation, making annual YAN measurement more important for quality wine production.

Table 3.10 Optimum prophylactic nitrogen additions by cultivar, and risk of deficiency or excess YAN based on probability plots.

Cultivar	Prophylactic YAN Addition (mg/L)	<140 mg/L	Mean YAN (mg/L)	Max YAN[a] (mg/L)	>400 mg/L
Cabernet Franc	140	0%	215	302	<1%
Chardonnay	75	5%	275	411	6%
Merlot	100	2.5%	232	325	< 1%
Pinot noir	65	5%	285	460	10%
Noiret	120	2.5%	284	426	5%
Riesling	140	0%	224	325	<1%
Traminette	95	1.8%	225	285	<1%

[a]Maximum likely YAN concentration based on the 95% confidence interval around the mean.

Conclusions

Preharvest YAN concentrations show a strong correlation to harvest YAN, and can be used to create linear regressions that provide a good estimate of harvest YAN in several wine cultivars grown in the New York Finger Lakes. These early predictions can allow winemakers to make timely decisions about nitrogen supplementation. YAN measurements taken as early as five weeks pre-harvest have been found to effectively predict harvest YAN, largely because PAN, which makes up 70% of total YAN in the cultivars studied, remains static. Additionally, population distributions can be used to estimate appropriate prophylactic additions for each cultivar, as well as the risk of deficiency or excess nitrogen resulting from a prophylactic addition. In the Finger Lakes, it appears that prophylactic additions may be appropriate in Cabernet Franc, Riesling and Traminette, as these cultivars are likely to be deficient, and

therefore run a low risk of excess nitrogen post-addition. Using the methods outlined above, similar predictive models can be calculated for grape cultivars grown in other regions.

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CHAPTER 4

METHOD TO DIVINE THE ORIGIN OF FERMENTATION DERIVED WINE AROMA COMPOUNDS USING ISTOTOPE RATIO MASS SPECTROMETRY

Abstract: The contribution of aroma compounds synthesized *de novo* from hexoses by yeast during fermentation has been investigated using gas combustion isotope ratio mass spectrometry (GC-C-IRMS). Uniformly labeled ^{13}C glucose was added as a tracer to Riesling must which was then fermented by *Saccharomyces cerevisiae*. Volatiles were extracted from the wine using dichloromethane, and the carbon isotope ratio of the volatile compounds was measured. The contribution of carbon from hexose is calculated by the amount of ^{13}C enrichment in the compound relative to the amount of ^{13}C added to the system. Hexanol was the only compound that appeared to be mostly (>90%) grape derived. Fusel alcohols and their acetate esters derived 75% or more of their carbon from hexose, medium chain fatty acids appeared to be 100% hexose derive, while isobutyric acid (branch chain) carbon was only about 50% from hexose.

Introduction

Over 100 volatile compounds have been identified in wine, of which about 70 appear to be critical for wine aroma(Ferreira 2010). A small subset of these key aroma compounds are so-called “primary” odorants detectable in the grape, e.g. rotundone (“black pepper” in Syrah) and methyl anthranilate (“grapey/foxy” in Concord) (Siebert et al. 2008, Nelson et al. 1977). However, many important wine aroma compounds are “secondary” – that is, they appear during fermentation either due to *de novo* biosynthesis by yeast or bacteria, or else through metabolism of grape-derived precursor compounds such as glycoconjugates (Baumes 2009). “Tertiary”

odorants may also arise during storage through several mechanisms, including acid-catalyzed transformations, oak contact, or microbial spoilage.

Establishing precursors or formation mechanisms for wine components during or after fermentation is of interest to wine researchers, as this can lead to improved strategies for controlling the concentrations of these compounds in finished wines. Frequently, this is accomplished by addition of a putative precursor to an appropriate media. For example, limonene and α-terpeniol were added to model wines to evaluate the potential of these compounds to serve as precursors for 1,8-cineole ("eucalyptus" odor)(Capone et al. 2011), and a glycoconjugate-enriched extract was added to a model juice media prior to fermentation to quantify the contribution of yeast metabolic activity to the release of volatile aglycones (Ugliano et al. 2006). The challenge to these approaches is that they require adding proportionally large amounts of substrate and that it is not possible to determine if the substrate was directly transformed into the product. As a result, it may not be evident if changes in wine compounds during fermentation result from direct transformation of the precursor, or result from induced changes in microorganism physiology. For example, the higher alcohols (fermentation derived alcohols with more than two carbons, e.g. isoamyl alcohol) and their corresponding acetate esters are important wine odorants, with the former associated with off-aromas at high concentrations and the latter credited with the fruity aromas common to young wines (Ugliano and Henschke 2009). Many of the higher alcohols are proposed to be formed during alcoholic fermentation by one of two pathways related to amino acid metabolism: i) catabolism of grape amino acids pathway, characterized by deamination of amino acids to α-keto acids followed by decarboxylation to an aldehyde, and finally reduction to a fusel alcohol ("Ehrlich pathway") (Hazelwood et al. 2008), or ii) production of α-keto acids during amino acid biosynthesis from

48 sugars, which may then be degraded to fusel alcohols as described before ("anabolic pathway")
49 (Ugliano and Henschke 2009). Nitrogen supplementation can alter fusel alcohol production
50 (Hernandez-Orte et al. 2006, Hernández-Orte et al. 2005, Garde-Cerdan and Ancin-Azpilicueta
51 2008), but it is not clear if changes in the Ehrlich or anabolic pathways, or both, are responsible
52 for this outcome. As a further complication, some higher alcohols (e.g. β-phenylethanol) exist as
53 glycoconjugates in grapes, which can serve as an additional sources of these compounds in wine
54 (Ugliano et al. 2006).

55 Stable isotope tracers can be used as an alternative to adding unlabeled substrates for
56 evaluating precursor-product relationships. Tracer studies have been widely used in food
57 chemistry, e.g. for studies of precursors of Maillard reaction products (Schieberle 2005), and
58 have occasionally been extended to studies of the fate and origin of wine components. For
59 example, deuterated isobutyric acid and ethyl isobutyrate spikes were used to evaluate wine
60 components could serve as precursors to these compounds in stored wines (Diaz-Maroto et al.
61 2005). However, stable isotope tracers must be added at a concentration of at least >0.5% (and
62 generally higher) of the endogenous concentration to yield detectable changes using a typical
63 GC-MS system (Brenna 1994), which may be prohibitively expensive and again risks perturbing
64 fermentation physiology. Radioisotopes can be employed at lower concentrations due to their
65 low natural abundance, and in wine [^{3}H]-malvidin glucoside has been used as a model to track
66 the fate of anthocyanins before and after fermentation (Zimman and Waterhouse 2004).
67 However, radioisotopes are rarely used as tracers in food chemistry studies, likely because of the
68 hazards associated with their use.

69 An alternative to conventional tracer experiments by GC-MS is high precision isotope
70 ratio measurements by gas chromatography combustion isotope ratio mass spectroscopy (GC-C-

IRMS). In GC-C-IRMS, organic compounds are combusted to CO_2 following their elution from a GC, and the isotopomers of CO_2 (m/z = 44, 45, 46) measured by IRMS for each peak (Brenna 1994). The major advantage of GC-C-IRMS for tracer studies is that it can achieve much higher precision than conventional GC-MS such that ^{13}C enrichments as low as ~0.1% atom percent excess (APE) can be utilized (Asche et al. 2003). GC-C-IRMS has been used for tracer studies across a number of disciplines where a sizeable endogenous pool of a potential precursor exists and large degrees of enrichment are not possible for economic or other practical reasons (Brenna 1994). For example, GC-C-IRMS has been used to monitor the fate and metabolism of dietary omega-3 fatty acids in animal studies (Goodman and Brenna 1992). GC-C-IRMS has been used for natural abundance studies of wine, primarily for studies on authenticity and adulteration. For example, $^{13}C/^{12}C$ ratios can be used to detect illegal addition of cane sugar (derived from a C4 plant) to grape juice (derived from a C3 plant) prior to fermentation, and in combination with D/H and 18O/16O ratios can serve as geographical fingerprints for wine provenance (Reid et al. 2006). GC-C-IRMS has also been used in conjunction with $^{13}CO_2$ labeling studies of growth chamber-grown grapevines to determine the timing of glycoconjugate precursors (Baumes et al. 2002), but to our knowledge GC-C-IRMS has not been used for tracer studies of wine fermentations or in related areas of food processing.

In this work, we report the use of GC-C-IRMS as a novel approach to follow the fate of a stable isotope tracer during alcoholic fermentation. Uniformly labeled [U-^{13}C] glucose was added to a grape must prior to fermentation at trace levels (0.01, 0.1, and 1% atom percent excess), and its contribution to a diverse range of fermentation volatiles (i.e. fusel alcohols, fatty acids, and their associated esters) was assessed. This technique could be utilized as a more

general approach for studying the origin and fate of compounds in food systems while minimizing tracer usage.

Materials and Methods

Fermentations. Fermentations were conducted using Riesling juice from sourced from the Cornell research vineyards in Lansing, NY during the 2010 harvest. 1000 lbs of fruit was received by the Cornell Vinification & Brewing Technology Laboratory where it was crushed, destemmed, basket pressed, and settled overnight at ambient conditions. Approximately 30 L were siphoned into two 20 L Nalgene™ containers (Thermo Fisher Scientific Waltham, MA) and frozen at – 20°C. Before use the juice was thawed in a 40°C water bath for 1 h and mixed by inversion. Glucose,fructose and yeast assimilable nitrogen (YAN) were quantified by enzymatic colorimetric methods using a Chemwell 2910 Multianalyzer (Unitech Scientific, Hawaiian Gardens, CA). To supplement the initial juice YAN content of 40 mg N/L, diammonium hydrogen phosphate (674 mg/L) and Fermaid K® (Lallemand, Santa Rosa, CA), (250 mg/L) were added to yield a final YAN concentration of 214 mg N/L. Yeast (EC1118®, Lallemand, Santa Rosa, CA) was rehydrated in 40°C spring water (Crystal Rock Watertown, CT) with 40 g/L GoFerm (Scott Laboratories Petaluma, CA). and inoculated into the juice (5 L) at a rate of 0.3 g/L. Aliquots of 541 g (500 mL) of inoculated must were then added to 1 L Pyrex® media bottles (Corning Inc. Tewksbury, MA). Uniformly-labeled [U-^{13}C] glucose (Cambridge Isotope Laboratories Tewksbury, MA) was then added to the fermentations at one of 4 levels: 1.0% atom percent excess (APE) (1.0150 g/ 500mL), 0.1% APE (0.1015 g/ 500mL) and 0.01% APE (0.0102 g/500 mL), and a control (no [U-^{13}C] glucose added), where APE is defined by eq1:

Equation 1 $$\boldsymbol{APE = 100(F_{HEXe} - F_{HEXn})}$$

Where F_{HEXe} is the atom fraction of the enriched hexose and F_{HEXn} is the atom fraction of the native hexose pool. F_{HEXe} is calculated by the amount of [U-^{13}C] glucose added to the system using the mass balance Eq 2, which can be rearranged to Eq 3.

Equation 2 $$\boldsymbol{F_{HEXe}m_{HEXe} = F_{ULG}m_{ULG} + F_{HEXn}m_{HEXn}}$$

Equation 3 $$\boldsymbol{F_{HEXe} = \frac{F_{ULG}m_{UGL}}{m_{HEXe}} + \frac{F_{HEXn}m_{HEXn}}{m_{HEXe}}}$$

For tracer level additions of [U-^{13}C]-glucose, F_{ULG} = 1, and the fraction (m_{HEXn}/m_{HEXe}) will be ~1, and the equation simplifies to:

Equation 4 $$\boldsymbol{F_{HEXe} = \frac{m_{ULG}}{m_{HEXe}} + F_{HEXn}}$$

Combining eqs 1 and 4 yields eq 5, a good approximation for the APE of the enriched hexose pool (APE_{HEX}) as a function of the amount of [U-^{13}C] glucose added to the fermentation and the original hexose concentration.

Equation 5 $$\boldsymbol{APE_{HEX} = 100\left(\frac{m_{ULG}}{m_{HEXe}}\right)}$$

Fermentations were carried out in triplicate for each of the four treatments (three tracer levels + one control). The fermenters were topped with a three piece airlock with floating bubbler (Buon Vino Manufacturing Cambridge, ON). Fermentations were conducted over a 10 day period at ambient temperatures in a dark cabinet, and were measured for residual sugar after all fermenters had stopped producing CO_2. At completion, all fermentations had residual sugar values of less than 0.5 g/L. After fermentations were complete 50 mg/L of SO_2 was added and the wine was frozen and stored at -20°C until further analysis.

Sample Preparation. Volatile components were extracted from wine using a method described by Ortega and others(Ortega et al. 2001). To a 15 mL screw capped borosilicate glass centrifuge tube, 4.5 g of $(NH_4)_2SO_4$, 3 mL of wine, 7 mL of water, and 0.2 mL of dichloromethane were added. The samples were mixed for 1 h using a carousel rotating at 20 rpm to invert the tubes, then centrifuged at 2500 g for 10 min. Approximately 1.5 mL of the emulsified bottom layer was recovered with a glass Pasteur pipet and transferred to a 2.2 mL microfuge tube and centrifuged for 4 minutes at 14,000 g. Finally, 100 µL of the dichloromethane layer was transferred to an Agilent GC autosampler vial with a 250 µL microvolume insert.

Quantification and Identification of Volatiles by GC-MS. Compounds in the DCM extracts were identified and quantified by gas chromatography – mass spectrometry (GC-MS). An HP 6890 GC with a split/splitless inlet (Agilent Technologies, Palo Alto, CA) and a autoinjector were coupled to an Agilent 5795 quadrupole mass selective detector (MSD) via a four way rotary valve, which permits diversion to the microcombustion reactor and IRMS. A 60m×0.32mm×0.25µm Aglient HP-INNOWax column (polyethylene glycol) was used. The GC conditions were: initial head pressure at 8.57 psi with a flow of 1.6 mL/min in constant flow mode, inlet at 250 °C, splitless injection, total flow at 84.8 mL/min. The oven parameters were: 35 °C (initial, hold 2 min) ramped to 40 °C (5 °C/min, hold 5 min), ramped to 200 °C (3 °C/min hold 5 min), and ramped to250 (25 °C/min hold 20 min). The MSD was operated with an ionization energy of 70 eV. The voltage of the electron multiplier was 1700 V. The scan parameters covered a mass range of *m/z* 33-300. Chemstation MSD (Agilent Technologies, Palo Alto, CA) was used for data processing. Compound identification was performed by matching retention times and mass spectral data of peaks to authentic standards. For quantification the area

158 of the corresponding peaks was normalized by that of an internal standard, 2-octanol and was
159 interpolated from a standard curve prepared in model wine using pure standards and calibration
160 curves for all 26 compounds had $r^2 > 0.99$. Quantifier ions and retention times for each
161 compound are listed in Table 4.1.

162 Ethanol was measured separately due to its co-elution with the solvent. A direct injection
163 method (Wang et al. 2003) was used to quantify ethanol where the wine was filtered through a
164 0.25um filter and 100 uL was diluted with 900 µl of water. The sample was injected in split
165 mode with a 50:1 split in a 250°C injector port. The oven parameters were: 35 °C (initial, hold
166 2min) ramped to 40 °C (5 °C/min, hold 5 min), ramped to 70°C (5 °C/min hold 5 min), and
167 ramped to 250 °C (25 °C/min hold 7 min).

168 **Carbon Isotope Ratio Analysis of Wine Volatiles by GC-C-IRMS.** Design and
169 operation of the GC-C-IRMS system is described in more detail elsewhere(Zhang et al. 2009).
170 Briefly, an HP 6890 GC with a split/splitless inlet (Agilent Technologies, Palo Alto, CA) and
171 autoinjector were coupled to a Thermo MAT 253 IRMS (Bremen, Germany) via a specially
172 designed combustion interface. The IRMS was tuned for high linearity, and operated at a source
173 pressure of 2.3×10^{-6} Torr, and a source potential of 9.5 kV with a measured sensitivity of 1150
174 molecules/ion. Data were collected and analyzed using ISODAT 3.0 (Thermo Scientific Bremen,
175 Germany). The GC column was connected to an online micro-combustion reactor via a four-way
176 rotary valve which permitted solvent diversion. The micro-combustion reactor was constructed
177 from a 30 cm×0.5 mm i.d. alumina tube hand-packed with three 20 cm×0.1mm wires (i.e. 1 Cu,
178 1 Pt, and 1 Ni wire). The tube was maintained at 950 °C using a 30cm Thermcraft tube furnace
179 (Winston Salem, NC). Water generated due to combustion was removed from the system using a
180 Nafion® water trap (dimensions = 10 cm×0.8mm i.d.) immediately following the combustion

furnace. The open-split consisted of a 1m×0.075mm capillary connected to the IRMS inlet at one end, with the other end directly inserted into the post-water trap transfer line. The same combustion interface design was used for all measurements. For analysis of wine volatiles excluding ethanol a 60m×0.32mm×0.25µm Aglient HP-INNOWax column (polyethylene glycol) was used. The GC conditions were: initial head pressure at 8.57 psi with a flow of 1.6 mL/min in constant flow mode, inlet at 250 °C, splitless injection, total flow at 84.8 mL/min. The oven parameters were: 35 °C (initial, hold 2 min) ramped to 40 °C (5 °C/min, hold 5 min), ramped to 200 °C (3 °C/min hold 5 min), and ramped to250 (25 °C/min hold 20 min).

Ethanol was measured separately due to its co-elution with the solvent. A direct injection method(Wang et al. 2003) was used to quantify ethanol where the wine was filtered through a 0.25µm filter and 100 µL was diluted with 900 µl of water. The sample was injected in split mode with a 50:1 split in a 250°C injector port. The oven parameters were: 35 °C (initial, hold 2min) ramped to 40 °C (5 °C/min, hold 5 min), ramped to 70°C (5 °C/min hold 5 min), and ramped to250 (25 °C/min hold 7 min). ISODAT 3.0 was used to calculate isotope ratios in delta notation; ^{17}O corrections were made using the Santrock and Hayes method(Santrock et al. 1985). Background was determined using the individual background determination with a 5-point moving linear regression, peak detection was set to a slope of 0.5 mV/s and peak end was 0.4 mV/s.

CO_2 gas pulses were admitted from a pressurized tank during each GCC-IRMS run for isotope ratio standardization, three at the beginning and three at the end. The isotope ratio of the CO_2 standard gas is traceable to the international standard Vienna Pee Dee Belemnite (VPDB) $R_{VPDB} = 0.0112372$). Isotope ratios are reported as $\delta^{13}C_{VPDB}$ defined as:

203 Equation 6 $$\boldsymbol{\delta^{13}C_{VPDB}} = \frac{(R_s - R_{VPDB})}{R_{VPDB}} \times \mathbf{1000}$$

204 **Statistical Analysis.** All linear regression analysis and ANOVA calculations were
205 carried out using MiniTab (Minitab, Reading, MA) statistical analysis software.

206 **Calculating percentage of carbon derived from hexose in volatile compounds**. The
207 observed APE of each volatile (APE_{obs}) was calculated from experimental data for each volatile
208 in each tracer fermentation, where R_E is the $^{13}C/^{12}C$ ratio in the enriched sample and R_N is the
209 mean $^{13}C/^{12}C$ ratio of the corresponding compound in the natural abundance
210 fermentations(Brenna 1994):

211 Equation 7 $$\boldsymbol{APE_{obs}} = \frac{R_E - R_N}{1 + (R_E - R_N)}$$

212 To determine the percent of each volatile compound that originated from hexose substrate,
213 (APE_{obs}) was assumed to be related to the APE of the hexose pool (APE_{hex}) by eq 8.

214 Equation 8 $$\boldsymbol{APE_{obs}} = \boldsymbol{\alpha}(\%\ \boldsymbol{from\ Hexose}) \times (\boldsymbol{APE_{HEX}})$$

215 $APE_{\mathbf{hex}}$ is a factor of the tracer level (Eq 5). The apparent fractionation factor, α, can arise from
216 kinetic isotope effects and potentially could vary among compounds. However, in most
217 biochemical processes, α is expected to range from between 0.98 and 1.02, and assuming α=1
218 will introduce negligible error. Eq 8 can be simplified and rearranged to solve for the % of
219 carbon in a given volatile derived from hexose sugars

220 Equation 9 $$\%\ \boldsymbol{from\ Hexose} = \frac{\boldsymbol{APE_{obs}}}{\boldsymbol{APE_{HEX}}}$$

221

222

223 **Table 4.1** Volatile Composition of wines reported in mg/L produced by fermentations with different levels of [U-
224 ^{13}C] glucose enrichment[a]

Compound	RT[b]	Qion[c]	Atom percent excess of hexose (APE_{hex})			
			0%	0.01%	0.1%	1.0%
Ethanol	na	na	114848	112466	114059	118289
Isobutyl acetate	10.591	43	0.14	0.11	0.09	0.08
Ethyl butyrate	11.571	71	0.23	0.25	0.21	0.19
Ethyl isovalerate	13.383	57	0.04	0.05	0.03	0.07
Isobutyl alcohol	14.297	43	30.70	28.25	27.77	26.25
1-Butanol	16.875	56	1.45	1.42	1.44	1.45
Isoamyl acetate	18.744	43	3.41	2.98	2.46	2.55
Isoamyl alcohol	20.300	55	74.07	102.70	84.88	81.49
Ethyl hexanoate	21.413	88	0.34	0.35	0.27	0.28
Hexyl acetate	23.332	43	0.15	0.17	0.18	0.15
Ethyl lactate	26.774	43	5.65b	16.97a	13.84a	12.45a
1-Hexanol	27.144	56	0.74	0.97	0.83	0.76
Ethyl octanoate	30.802	88	0.21	0.38	0.23	0.24
Ethyl 3-hydroxybutyrate	34.474	43	0.13	0.18	0.15	0.14
Propanoic acid	35.567	74	3.73	4.32	3.36	3.00
Isobutyric acid	36.723	43	2.11	2.29	1.82	1.51
Butyric acid	39.111	60	0.30	0.15	0.13	0.16
Ethyl decanoate	39.228	88	1.34a	1.98a	1.39	1.16
Isovaleric acid	40.685	60	0.64	0.86	0.75	0.65
Diethyl succinate	40.815	101	0.12	0.22	0.18	0.18
Methionol	42.374	61	1.59	2.60	2.23	1.96
Phenylethyl acetate	45.970	104	0.37	0.34	0.29	0.27
Hexanoic acid	47.107	60	3.57	4.52	3.84	3.36
β-Phenylethanol	49.401	91	15.88	22.19	18.82	18.92
Octanoic acid	54.254	60	5.82	6.19	5.68	4.52
Decanoic acid	60.752	60	2.78a	0.93b	1.09b	0.88b

225 [a]Numbers within a row with different with different letters are different using the Bonferroni family error rate alpa =
226 0.05; no letters are present then no significant differences were observed for that compound.
227 [b]RT = retention time in minutes; [c]QIon is the atomic mass used to quantify the compound.
228

229 **Results and Discussion**

230 **Volatile composition of fermentations.** A summary of the average concentration of
231 volatile compounds by level of enrichment is given in Table 4.1. Mean concentrations of each
232 compound were within the range of values reported previously in analysis of wine samples
233 (Ortega et al. 2001). Individual ANOVA were calculated for each of the 28 compounds by level
234 of enrichment. To account for potential type 1 error due to the number of comparisons the
235 Bonferroni correction using a family error rate of alpha = 0.05 was applied. Ethyl lactate, ethyl

decanoate, and decanoic acid show significant differences between the control fermentation and the enriched fermentations. These differences may have arisen because the control fermentation was conducted at a different time than the enriched fermentations, rather than an effect of enrichment itself. In particular, production of mid-chain fatty acids like decanoic acid and their corresponding ethyl esters are known to be affected by variation in oxygen status during fermentation (Zamora 2009). However, for all other compounds, the lack of significant differences indicates that labeling does not perturb the system.

Table 4.2 $\delta^{13}C$ values in natural abundance control fermentations. Results are for means and standard deviations of fermentation replicates (n=3)

Compound	N	$\delta^{13}C_{VPDB}$	SD	Group
Acetic acid	3	-13.482	2.252	A
Isobutyric acid	3	-17.781	3.779	A B
Decanoic acid	3	-22.843	1.839	B C
Isoamyl alcohol	3	-24.12	1.688	B C D
Hexanoic acid	3	-24.235	1.285	B C D
Isobutyl alcohol	3	-24.472	0.401	B C D
Octanoic acid	3	-24.625	1.187	B C D
Ethyl octanoate	3	-24.801	1.97	B C D
Ethyl lactate	2	-25.842	0.46	B C D E F
1-Butanol	3	-25.885	2.031	C D E
Ethyl hexanoate	3	-25.937	1.876	C D E
Ethyl 3-hydroxybutyrate	3	-27.016	2.742	C D E F
Ethanol	3	-27.471	0.099	C D E F
Isoamyl acetate	3	-28.582	2.145	C D E F
Hexyl acetate	3	-29.152	7.554	C D E F
Methionol	3	-29.402	0.585	C D E F
β-Phenylethanol	3	-31.286	0.857	D E F
Phenylethyl acetate	3	-33.967	1.663	F
1-Hexanol	2	-34.192	0.211	E F

[a]Compounds with different letters differ at $p < 0.05$ level (Tukey's test).

Native ^{13}C enrichment. Table 4.2 reports the native isotope ratios of 19 volatiles in the experimental Riesling wine, expressed as $\delta^{13}C$ values. Literature reports on compound specific isotope ratios of wine volatiles other than ethanol are relatively rare (Spitzke and Fauhl-Hassek 2010), and to our knowledge our work represents the largest number reported to date. Significant

differences were observed in natural abundance isotope ratios among volatile compounds, and $\delta^{13}C$ values ranged from -13 to -35‰. Fewer compounds were characterized by GC-C-IRMS than were quantified by GC-MS because the combustion step of GC-C-IRMS converts all compounds to CO2; thus co-eluting peaks cannot be resolved (Brenna 1994) as they can be in GC-MS through use of selective ion monitoring. Precision, calculated as the standard deviation (SD) of $\delta^{13}C$ values from fermentation replicates, ranged from 0.1‰ (ethanol) to 7.5‰ (hexyl acetate), with a mean value of 1.9‰. This precision is worse than benchmark values for high precision GC-C-IRMS, SD($\delta^{13}C$) < 0.5‰(Brenna et al. 1997), because it reflects biological variability among the fermentation replicates rather than analytical variability alone. We observed much better precision, SD ($\delta^{13}C$) < 1.4‰ on average for analytical replicates of the same fermentation samples. The higher precision observed for ethanol likely reflects the fact that it is the major fermentation product throughout alcoholic fermentation, and thus should be less prone to fractionation. By comparison, many of the other volatiles in Table 4.2 account for <0.1% of the initial hexose substrate, and thus may be more sensitive to slight changes in fermentation conditions among replicates.

The isotope ratio of ethanol ($\delta^{13}C$ = -27.47‰), was within the range typically observed for wine, which is slightly depleted compared glucose from C_3 plants (A. Hobbie and Werner 2004). This effect has been previously observed, and arises because ethanol is derived from the C-1, C-2, C-5, and C-6 positions of hexoses, which are depleted with respect to the C-3 and C-4 positions (Rossmann et al. 1991). Several short- and mid-chain fatty acids (acetic, isobutyric, and decanoic acids) were enriched with respect to ethanol, as has been observed by another group (Spitzke and Fauhl-Hassek 2010). These volatile fatty acids are produced by yeast towards the end of fermentation(Viegas et al. 1989). Their enrichment is likely a result of the lighter

isotopes of sugars being metabolized preferentially during fermentation (Hunkeler et al. 2001); resulting in enrichment of the hexose pool by the time the majority of these fatty acids are formed. Most other volatiles, including fusel alcohols, ethyl esters, and several acetate esters, did not differ significantly in isotope ratio from ethanol. The most depleted compound measured was 1-hexanol ($\delta^{13}C$ = -34.19‰). As discussed in the next section, this is likely because hexanol is primarily derived from the ^{13}C-depleted grape lipid fraction as opposed to being synthesized by yeast from sugars. Phenylethyl acetate was also significantly depleted with respect to ethanol, although the reason for this was unclear.

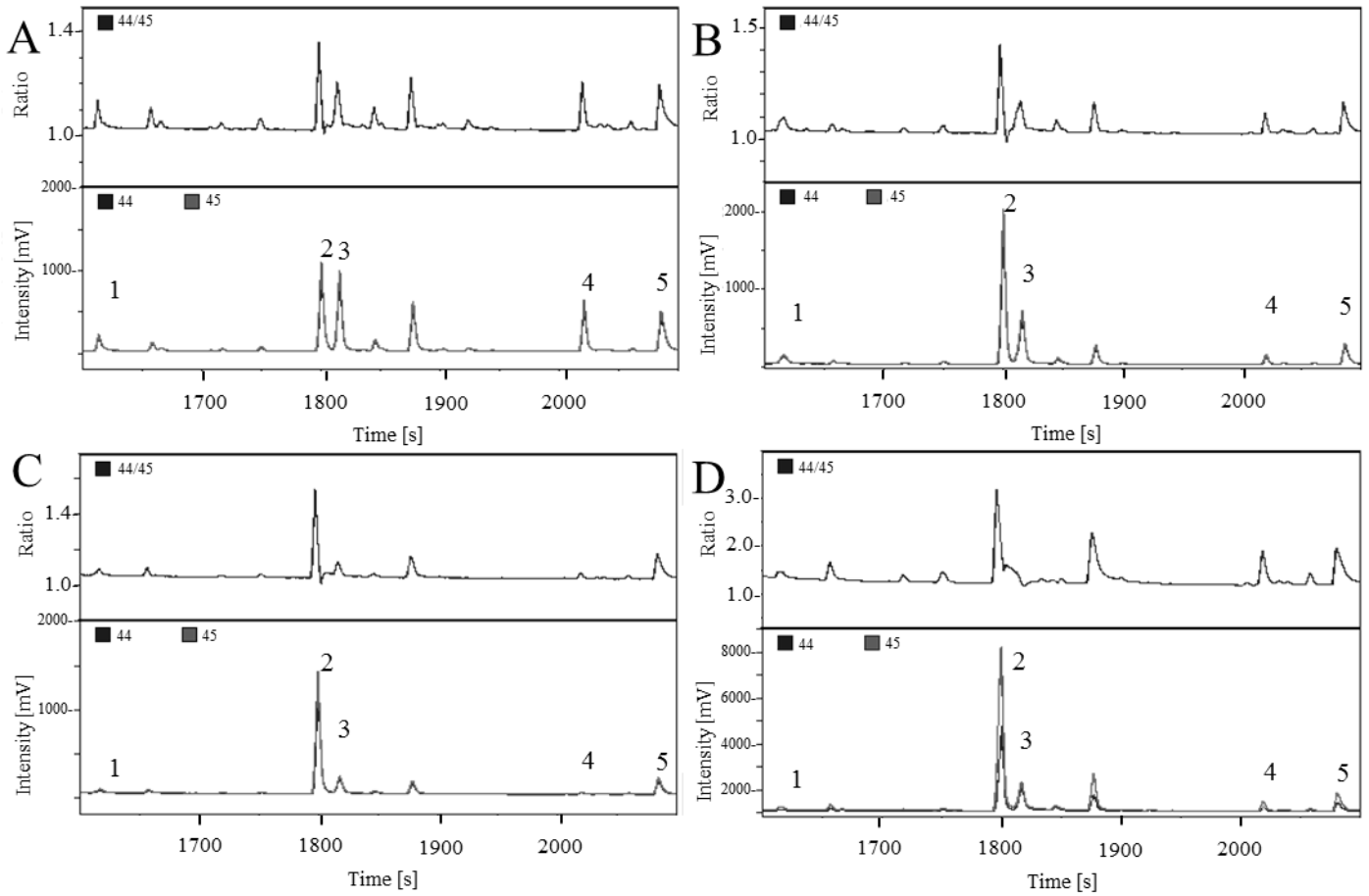

Figure 4.1 IRMS chromatograms of wine extract at different levels of ^{13}C enrichment: A control; B 0.01% [U-^{13}C] glucose; C 0.1% [U-^{13}C] glucose; D 1.0% [U-^{13}C] glucose. The 44 and 45 signals represent CO_2 ions containing ^{12}C and ^{13}C respectively from aroma compounds after separation and combustion. Peak identification: 1 hexyl acetate; 2 ethyl lactate; 3 hexanol; 4 ethyl octanoate; 5 acetic acid.

Determination of Hexose Contribution to Volatiles through Tracer Experiments.

Typical chromatograms obtained at different levels of [$U^{13}C$]-glucose enrichment are shown in Figure 4.1. With increasing enrichment of the hexose pool the *m/z* 45 trace increases relative to the *m/z* 44 trace for compounds such as ethyl lactate that derive most of their carbon from sugar. Conversely, the 45/44 ratio for compounds that are primarily grape derived, like hexanol, do not change visibly. APE_{obs} of volatile compounds in each fermentation experiment were calculated (Eq 7), and the percent of carbon derived from the hexose pool calculated by plotting APE_{obs} vs. APE_{hex} (Eq 9). Representative plots are shown in Figure 4.2 for octanoic acid, ethyl octanoate, ethanol, β-phenylethanol, and 1-hexanol, where the slope represents the percent of carbon within a compound derived from the hexose pool.

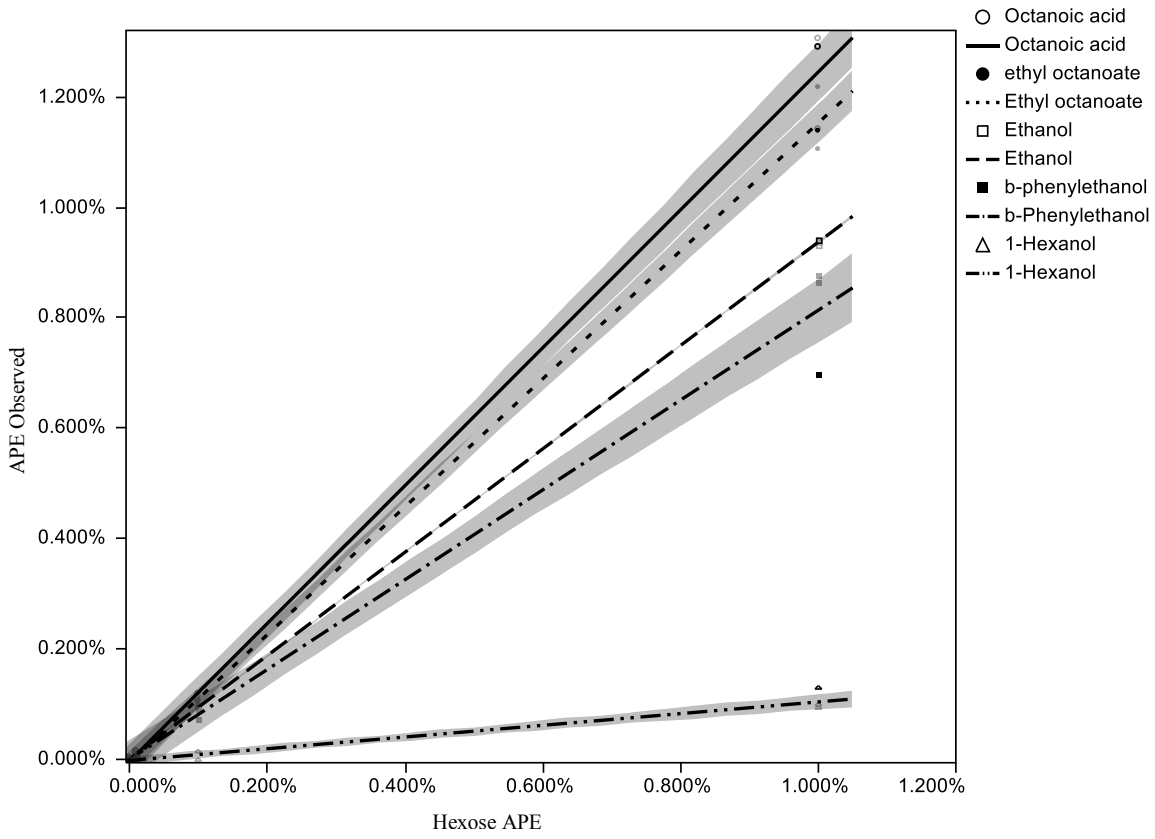

Figure 4.2 linear regression of observed APE vs. hexose APE. Shaded areas represent the 95% confidence interval.

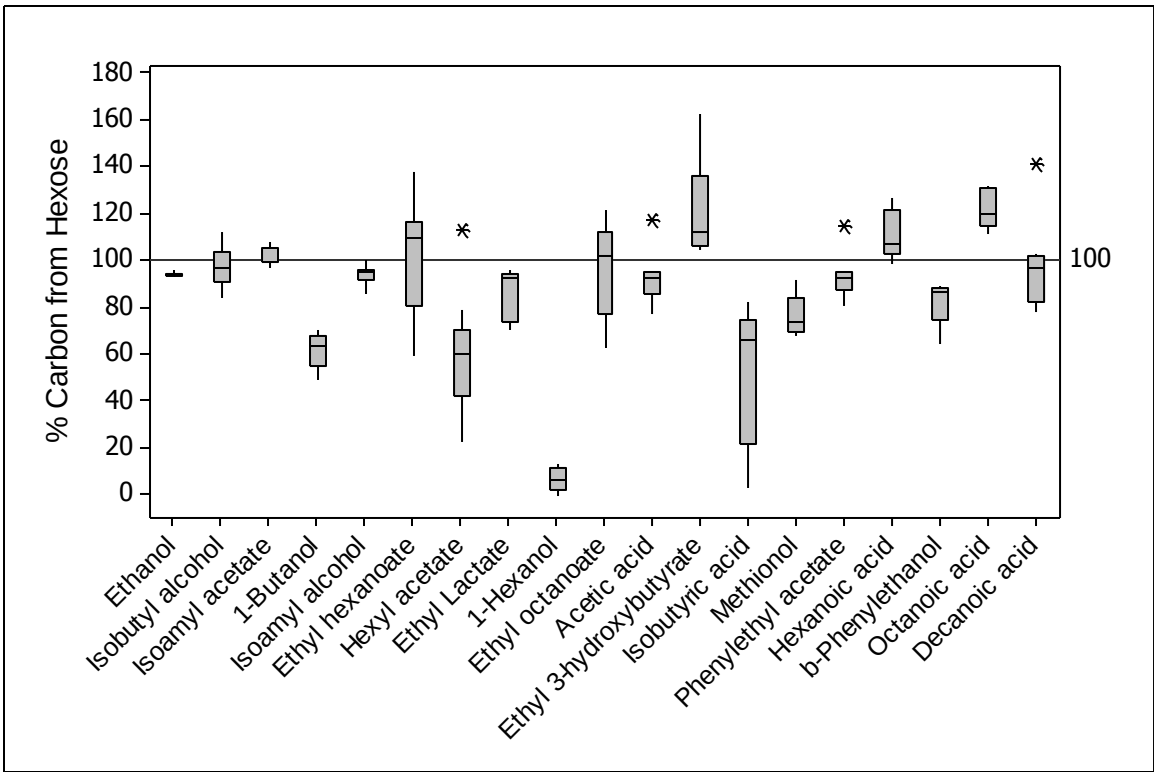

Figure 4.3 Variability associated with % carbon from hexose for each compound. Rectangular boxes represent the interquartile range of the data and the mean is indicated by the horizontal line inside the box; the lines extending from the box represent the upper and lower 25% of the distribution and the outliers are represented with an asterisk.

Table 4.3 and Figure 4.3 report the fraction of carbon derived from hexose in each volatile compound. The error associated with measurements of % hexose in individual compounds follow a similar pattern to what was observed in natural abundance studies. The contribution of the hexose pool to ethanol, which is present in large quantities and represents a major fermentation product, can be determined with high confidence (94 ± 0.5%), while the error associated with low concentration volatiles like ethyl 3-hydroxybutyrate are worse by an order of magnitude or greater.

The contribution of hexoses to ethanol (94%) was slightly lower than was initially expected (100%). Excluding the possibility of impure standards or experimental error, one likely explanation is that fermentable sugars other hexoses contributed to ethanol. In particular, sucrose

is reported to be present in musts at concentrations of 2-10 g/kg (Margalit, Yair,, Crum, James D.,Margalit, Yair,, 2004), or up to 5% of the hexose concentration in our work. Unfortunately, sucrose was not measured, and it was not possible to evaluate this hypothesis.

Table 4.3: ANOVA of percent of mass derived from hexose calculated by APE/% [$U^{13}C$] glucose[a]

Level	N	Mean	SE[b]	Grouping
Octanoic acid	9	122.3	2.81	A
Ethyl 3-hydroxybutyrate	9	121	7.39	A B
Hexanoic acid	9	110.8	3.35	A B C
Isoamyl acetate	9	103.2	1.31	A B C D
Ethyl hexanoate	9	102.1	8.24	A B C D E
Isobutyl alcohol	9	96.9	3.03	B C D E
Decanoic acid	9	96.8	6.27	B C D E
Ethyl octanoate	9	96.1	6.63	B C D E
Ethanol	9	94.2	0.26	C D E
Isoamyl alcohol	9	94.0	1.40	C D E
Phenylethyl acetate	9	93.1	3.19	C D E
Acetic acid	9	92.4	3.73	C D E
Ethyl Lactate	9	86.1	3.42	C D E F
β-Phenylethanol	8	81.9	3.16	D E F G
Methionol	9	77.4	2.90	E F G H
1-Butanol	9	61.5	2.50	F G H
Hexyl acetate	9	60.1	8.64	G H
Isobutyric acid	9	52.7	9.88	H
1-Hexanol	9	6.8	1.62	I

[a]Rows with different letters are significantly different using Tukey's test. $p < 0.05$ was considered significant.
[b]SE is standard error of the mean

Like ethanol, nearly all wine volatiles measured in our studies were derived primarily from hexoses (Figure 4.3). Of particular interest was that the fusel alcohols associated with amino acid metabolism (e.g. methionol, β-phenylethanol, isoamyl alcohol, isobutyl alcohol) were derived mostly, >75%, from hexoses. The acetate esters (isoamyl acetate, phenylethyl acetate) are formed by acetylation of fusel alcohols, and were also derived primarily from hexoses. This indicates that the major contributor to these fusel alcohols is the anabolic pathway, in which carbon skeletons are synthesized *de novo* from hexoses (Ugliano and Henschke 2009), as opposed to catabolism of amino acids via the Ehrlich pathway(Hazelwood et al. 2008). A third

potential source for β -phenylethanol is from juice in either a free or glycosylated form, although based on data from a previous report, this could only account for 3% of the total β -phenylethanol we observed (Ugliano and Moio 2008). To our knowledge, this is the first time that the relative contributions of different pathways to fusel alcohols have been evaluated. However, the conclusion that the anabolic pathway is dominant seems reasonable considering the amounts of fusel alcohols produced and the typical concentrations of amino acids in must available for Ehrlich degradation. For example, we observe *ca.* 100 mg/L of isoamyl alcohol in the wines, but typical leucine concentrations in juice are reported to be <25 mg/L (Ough et al. 1991).

The high contribution of the hexose pool to the other volatile compound classes, such as ethyl esters and fatty acids, was generally unsurprising, as many of these compounds are well-known to be produced by yeast metabolism (Ugliano and Henschke 2009). Isobutyric acid had roughly only half of its carbon derived from the hexose pool, significantly less than many of the short chain fatty acids (Table 4.3). Branched chain fatty acids have been shown to decrease with supplementation of must with ammonia salts (Vilanova et al. 2007), indicating they may arise from catabolism of amino acids (Styger et al. 2011). Potentially, this result indicates that a large portion of isobutyric acid arises from valine catabolism.

Only one compound, 1-hexanol, was derived primarily from non-hexose sources. Although hexanol is generally present at concentrations at or greater than 1 mg/L in wine, alcoholic fermentation of juice-like media in the absence of grape-derived compounds results in no detectable hexanol in resulting broth(Ugliano et al. 2006). Hexanol is detectable in grape must, and can also be produced during fermentation by the reduction of C_6 aldehydes and unsaturated C_6 alcohols (Herraiz et al. 1990). These C_6 compounds are largely derived from enzymatic

354 oxidation of unsaturated fatty acids during grape crushing (Joslin and Ough 1978). Unsaturated
355 fatty acids, along with other lipids, are known to be 13C-depleted in plants (Chikaraishi et al.
356 2004), a result which correlates with our previous observation that hexanol is depleted as
357 compared to other wine volatiles (Table 4.2). Hexyl acetate, comprised of hexanol and acetate, is
358 enriched to the level predicted by the mass balance of acetic acid and hexanol (Table 4.2).
359 **Enrichment Levels.** The precision of this method depends not only on the precision of
360 the analytical instrumentation, but also on the concentration of the compound of interest, the
361 amount of ^{13}C in the compound and variability of the biological system under study to
362 fractionate isotopes. The external precision of the GC-C-IRMS system can reliably obtain a
363 standard deviation (SD) $\delta^{13}C_{VPDB} < 0.4‰$(Brenna et al. 1997). However at natural abundance
364 the average SD $\delta^{13}C_{VPDB}$ was 1.8‰ and in fermentations enriched with 0.01%, 0.1%, and 1.0%
365 [$U^{13}C$] glucose the average SD was 1.7‰, 6.4‰, and 53.4‰ respectively, when looked at
366 relative to the increase in $\delta^{13}C_{VPDB}$ each have relative SD around 6%. Due to the high cost of
367 [$U^{13}C$] glucose in future studies 0.1% enrichment is the best balance of lower cost with low
368 variability.
369 **Conclusions**
370 This study presented a method utilizing GC-C-IRMS to trace the origin of aroma
371 compounds during the fermentation of grape juice into wine using by enriching the hexose pool
372 with ^{13}C. Addition of 0.1% [U-^{13}C] glucose provided a strong signal while minimizing the cost
373 of enrichment. The percent of carbon derived from hexose varies significantly in aroma
374 compounds found in wine. Under the conditions studied, aroma compounds derived at least 50%
375 of their carbon from hexoses indicating that they are produced de novo by yeast during
376 fermentation. Hexanol was the only compound that appeared to be mostly (>90%) grape

derived. The fusel alcohols methionol and β -phenylethanol appear to have a significant portion of their carbon derived from plant precursors other than sugar, the most likely source would be amino acids or glycosylated forms. Further study of the origin of aroma compounds under variable fermentation conditions, including temperature, Brix, and PAN and AMM concentrations using the strategy described here may provide more detailed information about how the fermentation environment affects biochemical pathways of aroma production. This method could also be applied to other complex food systems to trace the origin of compounds produced by complex pathways such as Mallard reaction products.

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CHAPTER 5

DO THIRD PARTY WINE RATINGS RUIN THE TASTE EXPERIENCE OF SUPERTASTERS?

Abstract

Cues such as wine ratings, label design, or the suggestions of wait staff can bias a consumer's enjoyment of wine, but are some consumers more susceptible than others? It may depend on taste and chemesthesis physiology – specifically perceived bitterness of 6-n-propylthiouracil (PROP) and the chemesthetic cooling effects of menthol. In this pilot study using dealcoholized wine as a model system, results suggest that PROP tasters were less influenced by third-party ratings than non-tasters, and panelists with higher response to menthol stimulus were more influenced than their counterparts with lower response. Despite their increased taste acuity, however, PROP tasters reported being significantly less confident when choosing a wine than non-tasters. However, a second experiment with more experienced wine consumers showed no significant differences in confidence or influence of external cues on panelists with different taste phenotypes. This confidence conundrum may be the result of incongruence between the preferences of inexperienced PROP tasters and those of the wine media, but with more experience this effect seems to diminish. This study serves as a stepping-stone for further work examining the relative importance of third-party ratings and wait staff suggestions on beverage sales.

Introduction

Naïve responses to foods and beverages are rarely objective. Consumer perceptions of taste, smell, and appearance are influenced by external cues, such as packaging, labeling, and the

opinions of others (Imm et al. 2012, Wansink et al. 2007). In wine, relevant external cues may include expert ratings, competition awards, label design, and bottle type (Gil and Sánchez 1997, Ortha and KrsÏkab 2002), all of which impact perception of wine sensory properties and shape subsequent consumer response and preferences. Such cross-modal interaction may have a powerful effect on product perception and experience (Spence 2011). Individual physiology also plays a role; taste phenotypes, and bitterness sensitivity in particular, result in widely variable gustatory response (Tepper 2008). The interaction between the sensory properties of beverages, external cues, and taste phenotypes has not been previously examined, and, if not controlled for, may complicate interpretation of consumer study data.

For decades, sensitivity to 6-n-propylthiouracil (PROP) has been used to determine "supertaster" status (Hayes and Keast 2011), as distinct phenotypes. Recently, differences in taster status were related to genetic differences in the *TAS2R38* bitter receptor (Kim et al. 2003). Using suprathreshold concentrations of PROP to determine phenotype, Hayes, Bartoshuk, Kidd, and Duffy (2008) found that 24% of the population studied could be described as nontasters, 54% as tasters, and 22% as supertasters. Polymorphism in the bitter receptor gene does not, however, fully explain supertasting. While TAS2R38 is the most studied, it is one of 25 unique bitter receptors in the TAS2R class of receptors (Hayes et al. 2013). Differences in taste phenotypes do not just exist for bitter sensations, alternate, non-PROP-based measures for identifying individuals with heightened response include irritant bitter tasting (iBT) and thermal tasting (TT), both of which are independent of PROP bitterness (Bajec and Pickering 2008, Green and Hayes 2003). While PROP supertasters show an increased perception of astringency and irritation (Pickering et al. 2004), these sensations are independent of bitterness perception, and are carried by the trigeminal nerve, rather than the glossopharyngeal nerve that transmits

bitterness (Barrett and Ganong 2010). The cooling sensation caused by mints is also PROP-independent, and is caused by stimulation of the touch nerve through menthol activation of temperature sensitive receptors (Bandell et al. 2007).

This variability in response to oral stimuli may impact response to external cues. PROP tasters have been known to have aversions to certain foods, especially cruciferous vegetables (Duffy et al. 2010, Tepper 1998), and it has been suggested that PROP tasters perceive red wines to be more bitter or irritating, while non-tasters perceive the same wine to be less bitter and more sweet (Duffy et al. 2004). Based on these observations, Pickering, Simunkova and DiBattista (2004) hypothesize that PROP supertasters may avoid wine styles that are high in bitterants or astringent compounds. As wine styles currently touted by wine 'experts' are often higher in these orosensory compounds, aversion to such compounds may lead to decreased wine consumption and lower confidence in wine selection in the supertaster population (Hanni and Utermohlen 2011).

To date, literature has focused primarily on the effect of PROP sensitivity on food choices, but recent work decoupling general supertasting from PROP supertasting (Reed 2008) and growing interest in cross-modal effects on consumer perception leads to questions about the interaction of menthol taste response (mediated by the trigeminal nerve) and PROP response (mediated by the glossopharyngeal nerve) with external product cues. This work describes two independent studies using a similar method to determine the influence of external cues, taste sensitivities, and multimodal interactions on consumer perception of wine.

Materials and Methods

Dealcoholized wine as a model system. The first study comprised fifty-two panelists, with 13 male and 39 female (average age 22.2 years) participating voluntarily in exchange for class credit. Panelists were asked to evaluate two dealcoholized wines, a dry Chardonnay and a sweet white Zinfandel, produced by St. Regis Vineyards, Madera, CA (Table 5.1) using one of three ballots. One ballot indicated that the Chardonnay had won an award and received a 92pt Wine Spectator score, one that the White Zinfandel had won the award, and the third lacked award information for either wine. Panelists were asked to taste the wines and rate them for overall liking on a 9-point Quartermaster hedonic scale. Sweetness and tartness were evaluated on 9-point just about right (JAR) category scales. The sweetness JAR scale was anchored with "Not very sweet" and "Extremely too sweet", and the tartness JAR scale with "Not very tart" and "Extremely too tart." Following the evaluation of the wines, subjects filled out a short survey asking about their consumption habits for coffee and alcoholic beverages, and confidence when choosing a wine.

After the wine evaluation and survey, subjects were asked to evaluate a breath mint, and were given the following instructions: "Please unwrap the mint and place it in your mouth. After about 15 seconds breathe in quickly through your mouth. Please describe the intensity of the cooling sensation by marking your response on the line provided." They were provided with a nine-point hedonic scale anchored with "Not very intense" "Moderately intense" and "Very intense."

In a separate session, 32 of the original panelists were given a 0.56 mM solution of PROP and asked to rate the overall intensity of the solution on a nine-point scale and record one word to

describe the taste quality, using a method described by Lawless (1980). The nine-point scale was anchored with "No Taste", "Moderate Taste" and "Very Strong Taste."

The project plan followed a 3x2x2 between-subjects design, in which the external stimuli (Chardonnay award, White Zinfandel award, no award) were randomly manipulated, and menthol response and PROP taster status were measured. Based on the bimodal distribution of mint intensities, subjects fell into two groups. Individuals who were grouped as having high response to menthol (n=20) had an intensity score of 5 or greater, while tasters labeled as having low response (n=32) rated the intensity of the mint as 4 or lower. Response to PROP also elicited a bimodal distribution with PROP tasters (n=23) having a score of 4 or greater and non-tasters (n=9) rating the intensity 3 or less.

MiniTab's General Linear Model (GLM) was used to model the effects of external stimuli, menthol response, and PROP taster status on the difference in liking score between the sweet wine (White Zinfandel) and dry wine (Chardonnay). A difference of $P < 0.05$ was considered significant.

Real wine, real wine consumers. A second study was conducted with 66 wine consumers, 27 male and 39 female, with an age range of 21 to 70 years and a mean age of 49 years. Panelists evaluated two wines, a sweet white wine (2010 Hermann J. Weimer Semi-dry Riesling, Seneca Lake, NY) and a dry red wine (2009 Chateau Briot, Bordeaux, France). The experimental design followed a 3x3x3 between subjects design where panelists received one of three ballots: one with no award, one indicating that the Riesling had won a Wine Spectator Award of Excellence, and one indicating that the Bordeaux had won the same award. Untrained panelists were asked to evaluate each wine for sweetness on a 7-point JAR scales anchored at "Not Sweet Enough" and "Much Too Sweet," and similarly-anchored scales for tartness and

bitterness. They were also asked to indicate their overall liking of the wine using a 7-point hedonic scale anchored at "Dislike Extremely," "Neither Like Nor Dislike," and "Like Extremely," followed with the forced choice "Circle the sample you preferred." Following the evaluation of the wines, subjects filled out a short survey asking about their consumption habits for coffee and alcoholic beverages, and their confidence when choosing a wine.

After the wine evaluation, panelists were given a 3.2 mM PROP solution to evaluate without swallowing (Hayes et al. 2008) on a labeled magnitude scale (LMS) with anchors at the following positions: 1= "Barely Detectable," 6= "Weak," 16= "Moderate," 34= "Strong," 51= "Very Strong," and 95="Strongest Imaginable" (Green et al. 1993). After a 5-minute forced break, panelists were given a 3% menthol solution (Cliff and Green 1994), which was evaluated on an identical LMS. Finally, after the wine evaluations panelists stained their tongues with blue #2 food dye using a cotton swab, and their tongues were photographed and papillae counted.

MiniTab's logistic regression (Logit) was used to model the effects of external stimuli, menthol response, and PROP taster status on the difference in liking score between the Riesling (sweet white) and Bordeaux blend (dry red wine). A difference of $P < 0.05$ was considered significant.

Results and Discussion

Dealcoholized wine main effects. There was no significant preference between dealcoholized sample wines (Table 5.1), though the sweet wine was viewed as significantly sweeter ($P<0.05$) and the dry wine significantly more tart ($P<0.05$). The main effects of menthol response and PROP taster status did not significantly affect overall liking for the dry or sweet product (Table 5.2). Similarly, ratings and awards showed no direct effect on preference for the

dealcoholized wines, suggesting that individual product preferences are not controlled solely by taste responses or the external award stimuli.

Table 5.1 Mean sensory attributes scores for dealcoholized wines (standard deviation in parenthesis)

	Chardonnay	White Zinfandel	T-Stat
Overall Liking	4.93 (2.19)	5.25 (2.09)	-0.76
Sweetness	5.30 (1.98)	6.20 (1.64)	-2.53*
Tartness	4.82 (1.98)	3.89 (1.85)	2.45*

*p<0.05
[a]Means were measured on a 9 point scale 1= Dislike Extremely, 9 = Like Extremely
[b]Attributes were measured on JAR scale 1= Not Enough, 5= JAR, 9 = Too much

Table 5.2 The impact of external information, menthol response and PROP taster status on overall liking of dry and sweet dealcoholized wines (standard deviations in parentheses)[a]

Dependent Variable	External Information			*P*-Value	Menthol Response		*P*-Value	PROP Taster Status		*P*-Value
	No Award	Dry Wine Award	Sweet Wine Award		Sensitive	Non-Senstive		Taster	Non-Taster	
Sweet Wine Liking	5.8 (2.2)	5.3 (2.3)	5.0 (1.9)	0.575	5.7 (2.1)	5.0 (2.1)	0.225	5.4 (2.3)	4.8 (2.2)	0.492
Sweet Wine Sweetness	6.2 (1.8)	6.1 (1.7)	6.3 (1.6)	0.909	6.1 (1.7)	6.3 (1.6)	0.604	5.8 (1.9)	5.9 (1.6)	0.873
Sweet Wine Tartness	4.4 (1.9)	4.4 (1.7)	3.3 (1.9)	0.116	3.9 (1.8)	3.9 (1.9)	0.883	4.2 (1.7)	3.3 (2.4)	0.325
Dry Wine Liking	5.5 (2.0)	4.7 (2.3)	4.9 (2.2)	0.683	5.3 (2.4)	4.7 (2.1)	0.432	5.0 (2.3)	5.4 (2.1)	0.698
Dry Wine Sweetness	6.1 (1.9)	5.0 (2.1)	5.3 (1.9)	0.365	5.7 (1.8)	5.1 (2.1)	0.299	4.8 (1.8)	4.9 (2.5)	0.909
Dry Wine Tartness	4.5 (1.9)	5.4 (1.8)	4.5 (2.1)	0.25	4.7 (2.0)	4.9 (2.0)	0.74	5.3 (1.7)	4.6 (2.1)	0.45
Liking Difference[b]	0.4 (2.2)	0.6 (2.1)	0.1 (1.6)	0.641	0.5 (2.4)	0.2 (1.5)	0.721	0.4 (1.9)	-0.6 (1.6)	0.172

[a] Liking was measured on a 9-point Quartermaster hedonic scale. Sweetness and tartness on a 9-point JAR scale: 1 = Extreme Low; 9 = Extreme High
[b] Liking Difference = Sweet Wine Liking – Dry Wine Liking

Main effects with standard wines: In the standard wine tasting, Riesling was preferred over the Bordeaux blend, and was deemed to be sweeter, less tart, and less bitter than the Bordeaux (Table 5.3). It was surprising that the Riesling was significantly preferred to the Bordeaux, as Mintel reported red wine is consumed slightly more than white wine overall (Bloom 2013), but this may be a result of regional bias towards Riesling, which is a popular variety produced in the Finger Lakes region of New York State. As with the dealcoholized wine, the main effects of external cue, menthol sensitivity, or PROP taster status did not have a direct effect on wine liking

(Table 5.4). However, as expected based on Pickering (2004), Supertasters did find the red wine to be significantly more bitter than did the non-tasters.

Table 5.3 Wine sensory attributes mean scores (standard deviation in parenthesis)

Wine	n	Riesling	Bordeaux	T-Stat
Overall Liking[a]	66	4.88 (1.28)	3.94 (1.47)	4.11*
Sweetness[b]	66	4.70 (0.98)	3.30 (1.08)	10.22*
Tartness[b]	66	3.74 (1.03)	4.53 (1.01)	-5.19*
Bitterness[b]	66	4.06 (0.89)	4.91 (0.99)	-6.05*

*p<0.05
[a]Means were measured on a 7 point scale 1= Dislike Extremely, 7 = Like Extremely
[b]Attributes were measured on JAR scale 1= Not Enough, 4= JAR, 7 = Too much

Table 5.4 The impact of external information, menthol response and PROP taster status on overall liking of Riesling (sweet) and Bordeaux (dry, bitter) wines (standard deviations in parentheses)[a]

	External Information			P-value	Menthol Response			P-value	Prop Taster Status			P-value
	No Award	Riesling Award	Bordeaux Award		Non-Sensitive	Sensitive	Very Sensitive		Non-taster	Taster	Super Taster	
	n = 22	n = 22	n = 22		n=11	n=33	n=22		n=20	n=30	n=16	
Riesling Liking	4.6	5.2	4.6	0.351	4.5	4.7	5	0.61	4.5	5.2	4.6	0.254
Riesling Sweetness	4.7	4.6	4.7	0.944	4.6	4.6	4.7	0.955	4.6	4.7	4.7	0.891
Riesling Tartness	3.1a	4.0b	4.1b	0.022	3.7	3.6	3.9	0.675	3.5	3.8	3.9	0.518
Riesling Bitterness	4	3.9	4.2	0.6	4.1	4.2	3.9	0.29	3.9	4.0	4.4	0.608
Bordeaux Liking	3.8	4.1	3.7	0.738	3.8	3.8	4	0.912	4.0	4.1	3.6	0.623
Bordeaux Sweetness	2.9	3.3	3.6	0.176	3.3	3.4	3.2	0.859	3.2	3.3	3.3	0.909
Bordeaux Tartness	4.9	4.4	4.7	0.501	4.9	4.4	4.7	0.39	4.8	4.5	4.6	0.814
Bordeaux Bitterness	4.8	5	4.7	0.599	4.5	5.2	4.8	0.129	4.8ab	4.4b	5.3a	0.034

[a] Liking was measured on a 7-point Quartermaster hedonic scale. Sweetness, tartness, and bitterness on a 7-point JAR scale: 1 = Much Too Low; 9 = Much Too High

Interactions of taste phenotypes and external stimuli and effect on preference. Despite the lack of significant preference for the dealcoholized wines studied, analysis of variance (ANOVA) results for the GLM suggest that interaction between an individual's taste responses and external stimuli may affect beverage preference (Table 5.5). The interaction between the external stimulus and menthol response approached significance (P=0.052), such that panelists

with a high response to menthol appeared to be influenced to a greater extent by external stimulus than those with a low response. For PROP tasters, the opposite trend was observed; the interaction between external stimulus and PROP taster status (P=0.083) suggests that PROP tasters were less influenced by award information than non-tasters. For this panel, the main effects of external stimuli (P=0.715), menthol response (P=0.793), and PROP taster status (P=0.484) were not found to be significant in driving liking differences between sweet and dry dealcoholized wines (Table 5.5).

Table 5.5 Analysis of variance of the general linear model for liking difference using adjusted SS for tests of main effects and interactions of external stimuli, PROP taster, and menthol response

Source	DF	Seq SS	Adj SS	Adj MS	F	P
External Stimuli	2	4.198	6.79	3.395	0.36	0.725
PROP Taster	1	5.418	7.721	7.721	1.03	0.484
Menthol Response	1	7.733	0.453	0.453	0.09	0.793
External Stimuli*PROP Taster	2	16.755	13.782	6.891	2.81	**0.083**
External Stimuli*Menthol Response	2	15.345	16.74	8.37	3.41	**0.052**
PROP Taster*Menthol Response	1	2.832	2.832	2.832	1.15	0.295
Error	21	51.575	51.575	2.456		
Total	30	103.855				

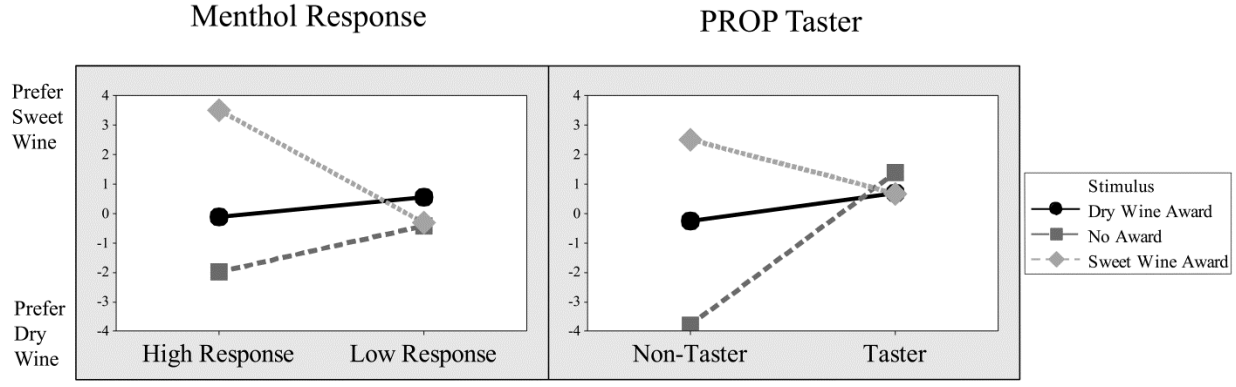

Figure 5.1 Interaction between external cues and taste sensitivity and their effect on differences in overall liking of sweet and dry dealcoholized wines

The relationship between external stimuli, response to menthol and the ability to taste PROP can be represented graphically using an interaction matrix (Figure 5.1). The graph on the left suggests that individuals with high response to menthol showed a wider range of differences between stimuli compared to individuals with low response. When no award was indicated, high-response menthol tasters preferred the dry dealcoholized wine to the sweet by about 2 units; in contrast, when the sweet wine was identified as an award winner, it was preferred by about 3.5 units. This suggests a stronger reaction to awards and ratings than that evinced by panelists with low response to menthol, where the difference in overall liking of the sweet and dry wines did not change with award status. The graph on the right of Figure 1 suggests that PROP tasters were less influenced by external stimuli, as the sweet wine had a higher overall liking score in all treatments. In contrast, non-tasters followed a pattern similar to the panelists with high menthol response, preferring the dry wine when no award was given and the sweet wine when it was identified as an award-winning product. If validated in further wine studies, these findings have potential implications for wine marketing, suggesting that advertising awards or expert ratings may not increase the perceived product liking among all taste phenotypes.

Interestingly, this interaction effect was not observed in the panelists evaluating wines. Using the forced preference data from the question "Which wine did you prefer?" a binary logistic regression showed no significance for any of the predictor terms in regards to preference (table 5.6). Directionally, however, the data is consistent with expectations. Panelists who saw the Riesling award were two times as likely to choose the Riesling than those who saw the Bordeaux award. PROP tasters and super tasters were more than three times as likely to choose Riesling as non-tasters. The lack of significance in the wine study may be a result of a more experienced panel; with a higher average age and two-thirds reporting weekly wine

consumption, these consumers were more likely to have developed preferences, less likely to be influenced by external cues.

Table 5.6 Logistic Regression of how external cues and PROP taster status affect the preference between Riesling (sweet white wine) and Bordeaux (dry red wine) wine.

Variable	Wine	Count	
Preferred sample	Riesling	47	(Event)
	Bordeaux	19	
	Total	66	

						95% CI	
Predictor	Coef	SE Coef	Z	P	Odds Ratio	Lower	Upper
Constant	0.405	0.913	0.44	0.657			
External Cue							
Riesling Award	0.693	1.225	0.57	0.571	2.00	0.18	22.06
Bordeaux Award	-0.118	1.190	-0.1	0.921	0.89	0.09	9.16
PROP Status							
SuperTaster	1.204	1.426	0.84	0.398	3.33	0.2	54.53
Taster	1.099	1.202	0.91	0.361	3.00	0.28	31.63
Ballot*PROP Status							
Riesling Award*SuperTaster	-1.204	2.008	-0.6	0.549	0.3	0.01	15.37
Riesling Award*Taster	-2.198	1.585	-1.39	0.166	0.11	0	2.48
Bordeaux Award*SuperTaster	0.118	1.954	0.06	0.952	1.13	0.02	51.77
Bordeaux Award*Taster	-0.134	1.634	-0.08	0.935	0.88	0.04	21.53

Test that all slopes are zero: G = 4.803, DF = 8, P-Value = 0.778

Influence of taste phenotypes and sex on beverage consumption and confidence.

Questionnaire responses for the dealcoholized wine study suggest that confidence in choosing a dealcoholized wine may be affected by taste phenotypes and sex (Table 5.7). The link between PROP phenotype and alcohol consumption reported in previous studies (Duffy et al. 2004) was not observed in this work, as no significant difference was found among groups for alcohol consumption. It's important to note, however, that about half of the panel (46%) was under legal US drinking age, and subsequently may have under-reported drinking habits, or may avoid alcohol consumption for legal, rather than physiological, reasons.

Table 5.7 Impact of sex, menthol response, and PROP taster status on beverage consumption and wine confidence. Means were measured on a 9-point scale (standard deviations in parentheses.)

Dependent variable	Sex		t-Stats	Menthol Response		t-Stats	PROP Taster Status		t-Stats
	Male (n=13)	Female (n=39)		Sensitive (n=20)	Non-Sensitive (n=32)		Taster (n=23)	Non-Taster(n=9)	
Coffee Consumption	3.1 (2.3)	4.9 (2.2)	-2.5**	5.1 (1.8)	4.0 (2.5)	1.91*	3.5 (2.5)	5.6 (1.7)	2.61**
Alcohol Consumption	3.9 (1.9)	4.4 (1.1)	-0.82	4.1 (1.5)	4.4 (1.3)	0.67	3.9 (1.2)	3.9 (1.8)	-0.04
Confidence	3.9 (1.1)	3.4 (0.9)	1.31	3.4 (1.1)	3.6 (0.0)	0.88	3.3 (1.1)	4.0 (0.7)	2.11**

*P<0.1
**P<0.05

Sex, menthol response, and PROP taster status may all affect frequency of coffee consumption. In the study with undergraduate subjects and dealcoholized wine, women consumed coffee more frequently than men (P<0.05), and non-tasters of PROP consumed more coffee compared to their taster counterparts (P<0.05). While this data suggests that heightened response to bitterness was related to decreased coffee consumption, other studies have demonstrated that consumption is based on more than taste factors alone; social, psychological, and economic factors also affect consumption behavior (Drewnowski et al. 1998, Lanier et al. 2005). It also appears that panelists with high response to menthol may drink more coffee than those who have a lower response (P<0.1). The lower rate of coffee consumption among PROP tasters may be due to their sensitivity to bitterness derived from components such as polyphenols (Bartoshuk et al. 1994). In the dealcoholized wine study, PROP tasters also self-reported lower confidence when choosing wines to share with others (P<0.05). It has been suggested that PROP tasters prefer wines that are sweeter and less bitter, preferences which conflict with wines preferred by experts; this disparity may cause tasters to question their own preferences (Hanni and Utermohlen 2011). In this study, however, tasters were not observed to have a significant preference for the sweeter dealcoholized wine. The lack of alcohol, and use of varieties with low polyphenol content (which enhances bitterness) (Robichaud and Noble 1990) may have been factors in this study.

The lower confidence of PROP tasters may also account for the lack of strong preference shown for either wine under any conditions. A recent study by Hayes and Pickering (2012) found that wine experts were more likely to be PROP tasters than other wine consumers, suggesting that, with experience, supertasters can develop a learned appreciation for a range of wine types, using their taste acuity to advantage in professional roles within the wine industry. This idea is borne out in the second study, where a more experienced panel of wine consumers reported no significant effects on confidence or consumption based on taste phenotypes (Table 5.8), suggesting that learned appreciation can overcome a phenotypic predisposition to dislike a food or beverage.

Table 5.8 Impact of sex, menthol response, and PROP taster status on beverage consumption and wine confidence. Means were measured on a 9-point scale (standard deviations in parentheses.)

Dependant Variable	Sex		p-value	Menthol Response			p-value	PROP Taster Status			p-value
	Male n=20	Female n=29		Non-sensitive n=11	Sensitive n=33	Very Sensitive n=22		Non-taster n=20	Taster n=30	Super Taster n=16	
Coffee Consumption	5.1 (1.4)	4.2 (1.2)	0.614	4.8 (2.1)	5.0 (2.3)	5.2 (2.1)	0.894	4.2 (2.4)	5.5 (2.0)	5.2 (2.1)	0.127
Alcohol Consumption	5.1 (1.4)	4.6 (1.2)	0.215	4.5 (1.4)	5.0 (1.3)	4.7 (1.4)	0.564	5.3 (1.0)	4.8 (1.5)	4.4 (1.1)	0.108
Confidence	5.5 (1.7)	4.3 (1.7)	0.02	5.2 (1.7)	5.1 (1.6)	4.6 (1.8)	0.528	5.1 (1.5)	5.2 (1.6)	4.3 (2.0)	0.252

Conclusions

These studies demonstrate the potential for exploring the influence of external cues, taste sensitivities, and multimodal interactions on consumer perception. Using non-alcoholic wine as a model and naive wine consumers as panelists, preliminary findings suggest that product preference can be influenced by the interaction between taste phenotypes and external cues, such as wine ratings. Namely, this work suggests three basic conclusions related to taste sensitivity:

- External cues have little impact on PROP tasters
- External cues have greater influence on those with a higher response to menthol

• Menthol response appears to be independent of ability to taste PROP.

However, the work with wine consumers evaluating commercial wines suggested that experience and familiarity can trump phenotypic predisposition, as interactions with taste phenotypes did not have significant effects on wine preferences or confidence.

This work suggests that phenotypes affect consumption habits and attitudes in naïve consumers. PROP tasters showed decreased consumption of coffee compared with non-tasters, and decreased confidence when choosing a dealcoholized wine. Menthol response did not appear to be related to consumption habits and attitudes as much as the ability to taste PROP, with only a moderate increase in coffee consumption among those with high menthol response, and no significant differences in alcohol consumption or confidence when choosing wine.

While external marketing cues are known to affect wine purchasing (Barber et al. 2006), data from this work suggest that such effects may not be equal across different taste physiologies or experience levels. In fact, attempts to compel consumers through marketing to buy a style of wine that is not congruent with their taste physiology may negatively affect their confidence in making future purchases, or may simply be ignored by more experienced tasters. Results from this pilot study suggest that the experimental method described above can be used to assess cross-modal influences.

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CHAPTER 6

CONCLUSIONS AND FUTURE WORK

Though nitrogen compounds in grape juice can affect fermentation parameters, including the type and amount of aroma compounds produced by the yeast, must YAN is rarely measured in wineries. Further, the origins of many fermentative-derived aroma compounds are not known. This research provides the enology and viticulture community and the scientific community at large with new methods for predicting YAN prior to harvest (Chapters II and III), determining the origin of fermentation-derived aroma compounds (Chapter IV), and assessing the interactions of taste phenotypes and external cues on the perception of wine (Chapter V).

YAN Prediction

The use of regression models and population data allow for better estimation of the concentration of YAN prior to harvest in multiple cultivars, including Cabernet Franc, Chardonnay, Merlot, Noiret, Pinot noir, Riesling, and Traminette. Early YAN predictions can allow winemakers to make timely decisions about nitrogen supplementation, thereby using less supplemental nitrogen while minimizing the risk of deficiency or excess resulting from a prophylactic addition. YAN measurements taken as early as five weeks pre-harvest have been found to effectively predict harvest YAN, largely because PAN, which makes up 70% of total YAN in the cultivars studied, remains static. In the Finger Lakes, it appears that Cabernet Franc, Riesling, and Traminette are chronically deficient in YAN and prophylactic additions may be appropriate in these cultivars with little risk of excess nitrogen post-addition.

Differences in nitrogen accumulation by cultivars were observed, and future research should be conducted to determine the causes of these differences and develop potential solutions to nitrogen deficiency. One aspect of nitrogen accumulation that deserves more research is

investigation of the flux of nitrogen. AMM concentrations decrease, but its fate is unknown. Does it enter the amino acid pool as proline? Is it incorporated into plant protein? A better understanding of the fate of nitrogen compounds in grapes may lead to better practices of cultivation and fertilization. The genes are associated with nitrogen deficiency in grape vines are also unknown; their discovery and could lead to the development of new varieties with lower risk of deficiency.

CG/C/IRMS and the Origin of Aromas

The application of GC/C/IRMS facilitated the investigation into the origin of fermentation-derived aromas. Enriching the hexose pool to 0.1% APE provided good precision while minimizing the cost of enrichment. Under the conditions studied, the aroma compounds derived most of their carbon from hexoses, indicating that they are synthesized de novo by the yeast from sugars. However the fusel alcohols methionol, b-phenylethanol and 1-butanol all derive at least 20% of their carbon from a source other than sugar. Based on existing knowledge of the Ehrlich pathway, the most likely source is amino acids. Consistent with previous reports, hexanol was observed to derive more than 90% of its carbon from grape sources other than hexose, most likely from C6 aldehydes and unsaturated alcohols.

The method described in this work for tracing the origin of aroma compounds can be employed to answer many more questions in the field of enology, and may find application in other food systems to trace the origin of synthesized aroma compounds. In wine, understanding how aspects of the fermentation environment like temperature, sugar concentration, nitrogen concentration, and other nutrients affect aroma production pathways would be the next step on this research path. Conducting fermentations with C^{13}-enriched glucose, and with varying levels of AMM and PAN, would be one way to study the effect nitrogen concentration has on the production of aroma compounds. This method could also be used to determine whether different yeast strains favor specific pathways for the production of aroma compounds. Understanding the

origins of aroma can allow producers to better control the type and amount of volatiles produced during fermentation to increase the quality and preference of their product.

Interaction Between Taste Phenotype and External Cues

Exploring the influence of external cues, taste sensitivities and their interactions on consumer perceptions holds great potential to influence wine marketing. Initial findings with naïve consumers and dealcoholized wine suggested that external cues have little impact on PROP tasters, while those sensitive to menthol are more influenced; further, these taste modalities are independent of each other. However, in a study with wine consumers experience appeared to be more important than phenotype, as differences in wine preferences or confidence were not observed. The decreased confidence observed in PROP tasters with little wine experience suggests that wines high in bitter and astringent compounds may not fit with the natural preferences of PROP tasters, and expert rating aimed at compelling them to purchase a wine may have negative effects. However, as a consumer becomes more experienced, the effect of external cues and taste physiology become less important to their wine choices.

Similar methods to investigate cross-modal influences may be used in the future to further investigate external cues and consumer perception; a few modifications, however, are necessary to ensure appropriate statistical power. Specifically, larger sample size and fewer experimental variables would increase the number of participants in each experimental condition, generating more robust conclusions. Future work may include genotyping participants to better understand the presence or absence of specific taste receptors. Additionally, better understanding of how the interaction between wine experience and taste physiology affect choices would be of interest.