Prediction of Prefermentation Nutritional Status of Grape Juice - The Formal Method

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1. Introduction

The Formol titration is a simple and rapid method for determination of the quantity of assimilable nitrogen in juice (1). It provides an approximate, but useful, index of must nutritional status. The procedure consists of neutralizing a juice sample with base to a given pH, adding an excess of neutralized formaldehyde, and re-titrating the resulting solution to an endpoint. The formaldehyde reacts with free amino groups of *alpha*-amino acids causing the amino acid to lose a proton which can then be titrated. Free ammonia is also titrated. Proline, one of the major amino acids in grapes which generally cannot be used by yeast under wine fermentation conditions, is partially titrated. Arginine, which contains four nitrogen atoms but only one carboxylic group, is titrated to the extent of the single acid functionality. Traditionally, barium chloride has been included to precipitate sulfur dioxide so that it does not interfere with the determination. If the juice is unsulfited or if the sulfur dioxide level is less than 150 mg/L, this part of the procedure may be ignored. (See note 8)

2. Analytical Methodology

2.1. *Materials*

Sodium hydroxide solution, 1N
Sodium hydroxide solution, 0.10 N, standardized against potassium hydrogen phthalate or equivalent
Barium chloride solution, 1N (0.05 Formula weight/L) (See note 8)

Formaldehyde, reagent grade, 37% (vol./vol. or 40% wt/vol.) neutralized to pH 8.0 with 1N sodium hydroxide
pH meter sensitive to ± 0.05 pH
Calibration buffers for the pH meter
Whatman No. 1 filter paper

2.2. *Method*

1. Pour 100 mL of sample into a 200-mL beaker.

2. Neutralize the sample to pH 8.0 using 1 N sodium hydroxide and pH meter.

3. If sulfur dioxide is present, add 10 mL of the barium chloride solution and allow the sample to sit for 15 minutes. (See note 8)

4. Transfer the treated sample into a 200-mL volumetric flask. Bring to volume with deionized water, and mix well.

5. Filter the solution through Whatman No. 1 filter paper.

6. Transfer a 100-mL aliquot of the sample into a beaker, place calibrated pH/reference electrodes and a stirbar into the solution, mix, and re-adjust the pH to 8.0, if necessary.

7. Add 25 mL of the previously neutralized formaldehyde (pH 8.0) to the aliquot, mix, and titrate to pH 8.0 using 0.10 N sodium hydroxide.

8. The concentration of assimilable nitrogen is calculated as follows:
   mg N/L (NH_4^+ + ∝amino nitrogen) = (mL of 0.1N NaOH titrated) x 28

2.3. *Notes*

1. The full equation for calculating assimilable nitrogen is:

   mg N/L = (mL of NaOH) x (0.10meq OH^-/mL)x(1meq N/meq OH^-) x (200mL/100mL-dilution factor)x 10 (to convert to liters) X 14 mg/mmol N
   = mL x 28

If a different concentration of base is used, the equation requires an additional term: (Normality of NaOH used/0.1). The dilution factor in the equation (200 mL/100 mL) is changed if one uses a sample volume other than 100 mL and dilution to 200 mL. Since there are ten 100 mL samples in a liter, the factor x 10 is required to convert results to a mg/L basis.

2. A new bottle of formaldehyde may have a pH as low as ~3.5. This will require about one-half milliliter of 1N sodium hydroxide to neutralize. If the formaldehyde is not neutralized, significant over-titrations may result yielding high values for fermentable nitrogen. The pH of the formaldehyde will begin to drop with time and should be periodically re-adjusted to pH 8.0.

3. Taylor (2) noted that various authors have recommended other pH values, ranging between 6 and 9, for the initial neutralization pH and final titration pH. It has been our experience with standard solutions of various amino acids that working with pH 8.0 for both the initial neutralization of the sample and for the final titration end point minimizes errors. The use of a pH meter for endpoint detection is an important feature of this method; the use of phenolphthalein as an indicator does not provide the same precision and accuracy.

4. Formol titrations of known concentrations of seven *alpha*-amino acids (alanine, arginine, serine, threonine, *alpha*-amino butyric, aspartic, and glutamic acid) and proline showed recoveries from 90 to 120% for the former and an approximate 17 to 33% recovery for proline. The percentage recovery appears to increase with the absolute amount of proline present. Similarly, Formol titration of known concentrations of ammonium chloride solutions (at approximately 50 and 100 mg/L nitrogen) also exhibited quantitative recoveries.

5. The arginine recovery is quantitative for the single carboxylic group. However, since arginine contains four nitrogen atoms, its recovery understates the amount of nitrogen present. Ingledew (3) reported that arginine converts to ornithine and urea, and that under the anaerobic conditions during fermentation, ornithine is almost quantitatively converted to proline. Thus, it would appear that arginine readily provides two assimilable nitrogens (in the urea formed) to the yeast, with a third nitrogen tied up as proline, and the fourth even-more-tightly-bound possibly to an enzyme system (Muller, personal communication).

6. Formol titrations of known mixtures of the eight amino acids mentioned in note 4 also showed nearly quantitative recoveries when the titration factors (percentages recovered) for proline and arginine were considered. The Formol method, therefore, overstates the available nitrogen from proline and understates the available nitrogen from arginine. The positive and negative errors introduced with the titration of these two juice components are partially compensating. If the amount of proline in the must is much larger (~ 10 times) than the amount of arginine, the method will overstate the amount of available nitrogen. If the amount of proline is only double the amount of arginine, then the positive and negative errors in the titration essentially balance out.

7. Under oxidative conditions proline is oxidized to glutamic acid and becomes available to the yeast, and the Formol method will generally understate the ultimate amount of nitrogen available.

8. A series of samples of a juice with 0, 25, 50, and 150 mg/L SO_2 added were titrated using the Formol procedure but ignoring the addition of barium sulfate. The average titration values determined for the various levels of SO_2 addition differed by 5.3% or less. This would indicate that it is not necessary to add barium sulfate to a sulfited juice sample. This would also simplify the procedure permitting one to take a 50-mL juice sample (50 mL) directly for titration.

9. Direct titrations of 25-mL samples of juice with correspondingly smaller amounts of formaldehyde added were made. Other than the greater difficulty in accurately reading small volume increments from the burette, there was no significant impact on the procedure.

3. Practical Considerations and Recommendations

Fermentation problems may arise from numerous sources including deficiencies in the fruit and processing (Figure 1). Difficulties may arise from a combination of factors and a variety of sources. It is often the impact of two or more conditions that cause a problem of greater difficulty than would be predicted by a single parameter alone. Once yeast fermentative vigor and vitality have diminished, revitalization may be difficult, if not impossible. Thus, winemakers must approach each winemaking step with as complete an understanding as possible. The following is a review of practical issues influencing fermentation.

3.1. *Vineyard*

Fermentation problems are often vineyard-specific. Nitrogen deficiency in apparently healthy grapes can be severe. Drought, grapevine nutrient deficiencies, high incidences of fungal degradation and level of fruit maturity all influence must nitrogen and vitamins. Cultivar, rootstock, crop load and growing season may also influence must nitrogen. Some varieties, such as Chardonnay, have a greater tendency towards deficiency. Higher total nitrogen may also be associated with certain rootstocks. For example, grapes grown on St. George are higher in total nitrogen than those on AXR1.

As seen in Figure 2, the concentration of *alpha*-amino nitrogen in Cabernet Sauvignon grapes changes as a function of maturity and crop load. Henick-Kling, *et al*. (4) compared the concentrations of the two important sources of assimilable N (FAN and NH_4^+) among six

cultivars at maturity over two seasons (Table 1). This study illustrated large variations from one season to the next in both free ammonia and *alpha*-amino nitrogen and significant differences in the concentration of both sources of nitrogen among cultivars.

Mold growth on fruit has been reported to cause fermentation problems due to the production of metabolites and the depletion of nitrogen (5,6,7). *Botrytis cinerea* produces a group of mold-derived heteropolysaccharides collectively referred to as "Botryticine" (8). The mycotoxins stimulate *Saccharomyces* sp. to produce high and inhibitory levels of acetic acid at the onset and during the latter stages of alcoholic fermentation (7). *Botrytis cinerea* can consume 41% of the total amino acid concentration in the fruit, causing as much as a 51% reduction in proline (9). Further, the presence of native yeasts, particularly *Kloeckera apiculata,* is known to deplete important vitamins such as thiamine. Fruit from diseased vines may also contain inhibitory levels of phytoalexins produced by the plant in response to the parasite (10). These may be inhibitory towards *Saccharomyces* sp.

3.2. *Yeast Strains*

Strain differences among *Saccharomyces* sp. may be significant in terms of nitrogen requirements, the time frame for uptake and release of specific amino acids during fermentation, and the ability to ferment to dryness. Henschke and Jiranek (11) reported that the Montrachet strain had the highest nitrogen demand and exhibited the highest rate of amino acid and ammonium ion accumulation relative to sugar fermented among several strains studied. When considering utilizing unfamiliar strains, the winemaker is urged to consult the supplier's technical representatives.

3.3. *Yeast Starter Population Density*

Yeast populations should be large enough to overwhelm indigenous microflora and grow to 2 to 5 x 10^6 yeast cells/ mL juice (1% to 3% vol/vol of an active starter). These concentrations apply when the ^{o}Brix is below 24, the pH is above 3.1, and the temperature is above 13^o C/55^{o}F. Increases in the inoculum volume should be made when parameters are outside these values.

3.4. *Yeast Preparation*

Rehydration protocol should strictly adhere to the yeast supplier's recommendations to assure maximum viability. Viability and vigor decrease as rehydration temperatures vary above or below those recommended. After rehydration the yeast should be added to the juice/must within 20-30 minutes or, alternatively, a carbohydrate source added. If this is not done, yeasts undergo a premature decline phase resulting in an inoculum of low viable cell density. Significant yeast cell death occurs when temperature differentials between starter and juice/must are more than 5-7^{o}C. Monk (12) reported that the addition of rehydrated yeast (40^{o}C /104^{o}F) directly to a must at 15^{o}C/60^{o}F kills approximately half of the cell population. In cases where yeast are expected to ferment at low temperature, it is desirable to acclimate the starter to that temperature.

3.5. *Nutrient Addition*

Although some have suggested that a minimum of 140 mg/L assimilable nitrogen is required by yeasts, others recommend 250 mg N/L or more. Morris, *et al.* (13) suggested that concentration levels of 500-900 mg/L of assimilable nitrogen are required for healthy fermentations. Yeast with lower concentrations of N may perform well under optimum, but not

adverse, conditions. Concentrations of 500-900 mg N/L give yeast the ability to produce cellular proteins needed to meet the worst environmental conditions (Cone, personnel communications, 1996). Many juice/musts lack sufficient assimilable nitrogen and other components needed by yeast for fermentative growth. Phosphate deficiency may also have a direct impact on yeast cell growth and fermentative performance (11). Inorganic phosphate is required for synthesis of ATP and ADP and nucleic acids. Supplementation should be carried out using a balanced source of diammonium phosphate, DAP (25.8% ammonia, 74.2% phosphate), amino acids, minerals, and vitamins. Diammonium phosphate additions of 1 g/L (8.3 lb/1,000 gal) provide 258 mg/L fermentable nitrogen which exceeds the suppliers' recommended level. In the U.S. the legal limit of DAP is 960 mg/L which corresponds to 203 mg N/L.

3.6. *Timing of nutrient additions*

Amino acids are not incorporated equally by yeast and may vary significantly among yeast strains (14). Some are utilized at the beginning of the growth cycle, some later, and some not at all. Ammonia, on the other hand, is consumed preferentially to amino acids in growing populations. Stationary phase yeast also vary significantly in terms of the order of amino acid incorporation, but do not always show the preference for ammonia over amino acids (14). Therefore, timing of DAP additions is important. A single large addition of DAP at the beginning may lead to an excessive fermentation rate and an imbalance in the uptake and usage of amino acids. To avoid this problem, multiple additions at 16^{o} Brix and 10^{o} Brix are preferred.

While addition of ammonium salts may not significantly benefit stationary phase yeast (15), the addition of specific amino acids may have a stimulatory effect and extend fermentative activity (16). Single amino acids may be quickly utilized to resynthesize transporter proteins that

are rapidly “turned over” during accelerated growth. Supplements added after about half the fermentation is completed may not be used by the yeast because alcohol prevents their uptake. For the same reason, adding nutrients to a stuck fermentation is seldom effective. With increasing ethanol concentrations the permeability of the plasma membrane to hydrogen ions increases. This requires intracellular enzymes and ATPases to pump protons back out of the cell in order to balance the internal pH of the yeast cell against the external pH of the juice/must. Due to the competing nature of these coupled transport systems, nitrogen is picked up by the cell only in the early stages of fermentation. It is stored in vacuoles and used upon demand. Nitrogen added late in the fermentation late cannot be transported into the cell (17). Once stopped due to nutrient stress, the fermentation may require significant effort to complete.

3.7. *Vitamin Addition*

Juice/must can be vitamin deficient as well as deficient in assimilable nitrogen when there is a high incidence of microorganisms (mold, yeast and/or bacteria). Growth of *Kloeckera apiculata* has been reported to rapidly reduce thiamine levels below those required by *Saccharomyces* sp. (18). Further, the use of SO_2 may lead to additional reductions in levels of thiamine (15). *Saccharomyces* sp. has been shown to synthesize all required vitamins, with the exception of biotin. However, vitamin supplementation has been demonstrated to be stimulatory (19). Thus, it is usually desirable to add a mixed vitamin supplement with the nitrogen additions.

3.8. *Yeast Hulls*

Yeast hulls are byproducts of the commercial manufacture of yeast extract. Consisting of cell walls and membranes, hulls are added to enhance fermentation rates and to restart stuck fermentations. Their mode of action has been described as lowering the concentration of inhibitory C_{8-10} fatty acids. Ingledew (3) reported that yeast hulls stimulate yeast populations by providing a source of C_{16} and C_{18} unsaturated fatty acids which act as oxygen substitutes under long-term fermentative conditions. Additionally, hulls may provide a source for some amino acids as well as surface area to facilitate release of potentially inhibitory levels of saturated CO_2.

3.9. *Oxygen/SO_2*

Although not directly stimulatory to fermentation, oxygen is required by yeasts for synthesis of cell membrane precursors including steroids (primarily ergosterol) and lipids (principally oleanoloic acid). Yeast propagated aerobically contain a higher proportion of unsaturated fatty acids and up to three times the steroid level of anaerobic yeast. Without initial oxygen, replication is usually restricted to 4-5 generations as each yeast budding cycle reduces the sterol content of the membrane by approximately half. When the level reaches a critical point, replication stops and fermentation must continue with the population present at that point.

The grape itself may supply at least a portion of the lipids needed by yeast during anaerobic growth. Up to two-thirds of the cuticular waxes in some grape varieties are composed of oleanolic acid. This fatty acid has been found to replace the yeast's requirement for ergosterol supplementation under anaerobic conditions (20). Thus, pomace contact, either prior to pressing in white wine production or extended during red wine fermentation, extracts this and other essential components from the grape cuticle.

Slight aeration of yeast starters may play an important role in subsequent fermentative performance. Wahlstrom and Fugelsang (21) reported increased cell density and more rapid fermentations when aerated starters were used compared with non-aerated starters. In the absence of sulfur dioxide, grape-derived oxidative enzymes (tyrosinases) catalyze conversion of nonflavonoid phenols to their corresponding quinones. The reaction brings about rapid (but reversible) browning of the juice while consuming oxygen required by yeasts during the early stages of growth (Figure 3). Grape tyrosinase is easily inactivated by addition of SO_2 to the juice/must. However, sulfur dioxide addition also inactivates thiamine. If additions of more than 50 mg/L SO_2 occur, thiamine (in the form of nutritional supplements) should be added to the fermenter.

3.10. *Hydrogen Ion Concentration (pH)*

Yeast growth occurs over the pH range from 2.8-8.0 (19). However, cultures do not function equally well throughout this wide range. Biomass is produced best above pH 4.0 and slows as pH goes down. Low pH reduces the tolerance of *Saccharomyces* sp. to ethanol. Kudo, *et al.* (22) demonstrated a relationship between the concentrations of K^+ and H^+ and the completion of alcoholic fermentation. They suggested that a minimum K^+/ H^+ of 25:1 is required. As pH drops below 3.2, the increase in H^+ raises the risk of premature arrest of fermentation. Added stress is placed on yeast at low pHs and is compounded by low nutrient concentrations, temperature extremes, high sugar and/or high alcohol. Additionally, highly chaptalized juice has a limited buffering capacity. As a result, the organic acid and CO_2 production during the initial stage of fermentation can drop the pH (Cone, personal communication, 1995). Juice/musts with pH <3.1 should receive an increased yeast inoculum.

3.11. *Nonsoluble Solids*

Nonsoluble grape solids serve as nutritionally important substrates and as oxygen reservoirs during the early stages of fermentation. Additionally, solids "hold" yeasts (native and inoculated strains) in suspension during the early stages of fermentation and before the evolution of large amounts of carbon dioxide. Conventional white juice processing calls for some level of suspended solids reduction prior to inoculation. However, reduction below 0.5% can result in nutrient deficiencies and promote premature sedimentation of yeast. Addition of bentonite may help to keep yeast in suspension during the initial stages of fermentation while helping to achieve protein stability. However, bentonite additions can also reduce must nitrogen and should be done in conjunction with supplemental nutrient additions. If processing protocol does not include pre-fermentation bentonite additions, it may be necessary to mix tanks to achieve resuspension and dissipation of carbon dioxide.

3.12. *Fermentation Temperature*

Yeast growth at either end of the recommended temperature range affects the integrity and operation of the cell membrane. Growth at upper temperature limits brings about inactivation/denaturation of cell membrane-associated transporter proteins and other enzymes whereas at low temperatures, fluidity/pliability is compromised (23). Cell membrane function is also affected by the presence of increasing concentrations of ethanol. The two antagonists act in synergy, narrowing *Saccharomyces* sp. temperature tolerance range and, potentially, bringing about premature interruption of fermentation. For low ($<10^{o}C/50^{o}F$) temperature fermentations, increased inoculum levels and nutrient additions are recommended.

3.13. *CO_2 toxicity*

Carbon dioxide in concentrations of up to 0.2 atm stimulates yeast growth. Above this level carbon dioxide becomes inhibitory. Pekur, *et al*. (24) reported that, at increased pressures, carbon dioxide reduces the yeast's uptake of amino acids. Agitation can be used to help prevent supersaturation of CO_2.

3.14. *Sugar toxicity*

Increased osmotic pressure associated with high sugar concentrations can inhibit yeast growth. Although *Saccharomyces* sp. are among the most tolerant species of high sugar concentrations, such environments are often nitrogen deficient. Fermentation under these conditions begins slowly and may stick prior to completion. In cases where sugar levels range from 25-30 ^{o}Brix, yeast starters should be prepared at greater than 5 x 10^6 yeast cells/mL. For >30 ^{o}Brix musts, an additional 1 x 10^6 yeast/mL should be used. Ice wines and some late harvest wines require substantially more yeast inoculum, up to 20 x 10^6 yeast/mL (Cone, personal communication, 1996).

3.15. *Glucose/Fructose*

Grape juice usually contains approximately equivalent concentrations of glucose and fructose sugars. However, glucose is fermented preferentially to fructose. Stress can affect the yeast's ability to metabolize the last residual fructose. This problem seems to occur more with the *S. bayanus* strains which are more glucophilic (25). Fructose syrup should be used only as the last choice for chaptalisation.

3.16. *Alcohol toxicity*

Alcohol and its metabolic precursor acetaldehyde are toxic to all yeasts, including *Sacccharomyces* sp. Alcohol has a profound effect on all aspects of yeast metabolism, ranging from membrane integrity to nitrogen uptake and sugar transport. There are many environmental factors that act in synergy with alcohol to inhibit yeast growth including low pH, high temperature, acetic acid, sugar, short chain fatty acids, nitrogen depletion, and deficiency of sterols and vitamins. Acetaldehyde has also been reported to play a significant inhibitory role in survival of *Saccharomyces* sp. during fermentation (26) and may increase the yeast's sensitivity to increasing concentrations of ethanol (27). Light aeration during the growth phase stimulates synthesis of cell membrane precursors which helps maintain cell integrity. During fermentation, nitrogen supplementation of 250-500 mg /L is likewise helpful in mitigating the antagonistic affects of alcohol.

3.17. *Microbially-compromised fruit, native yeast/bacterial fermentations, and late starter addition*

Usually non-*Saccharomyces* species from the vineyard and winery-associated *Saccharomyces* sp. dominate the initial and early stages of fermentation of uninoculated musts. Their growth may result in significant depletion of nitrogen and vitamins such as thiamine. Among vineyard-related native species, *Kloeckera/Hanseniaspora* are typically found at highest population densities. *Kloeckera* sp. are tolerant of both low temperature and the presence of sulfur dioxide. The yeast can produce high levels of ethyl acetate while significantly depleting nutrient levels.

Inhibitory metabolites produced by mold and native yeast/bacteria growing on fruit or in the early stages of fermentation may have a significant effect on the fermentative performance of

Saccharomyces species. Acetic and lactic acid bacteria and native yeast can produce potent inhibitors and deplete must nitrogen and vitamins levels. Acetic acid is a strong inhibitor of *Saccharomyces* sp. especially when combined with other antagonistic factors like high alcohol. Acetic acid levels of >0.8 g/L in stuck wine may need to be reduced before attempting refermentation (28). The technology to accomplish this goal is commercially available (29).

Some *Saccharomyces* sp. and strains and some non-*Sacccharomyces* yeasts can produce killer toxins that inhibit other sensitive strains and may play a role in stuck fermentations. It is suggested that vigorous strains be used for high risk fermentations. Increasing the level of yeast inoculum along with nitrogen supplementation of 250-500 mg/L may also help overcome these effects.

3.18. *Pesticides and Fungicides*

Pesticides and fungicides can influence fermentation by producing stress metabolites, such as reductive compounds, and by inhibiting and/or preventing fermentation. Not all yeasts and bacteria are affected in the same way. For example, there is a significant difference between systemic and contact fungicides with regard to residues. Vinification style influences residue concentrations. Pre-fermentation clarification and utilization of bentonite can affect the final concentration of contact fungicides in white wine fermentation. Close adherence to spray schedules, use of minimal applications and avoidance of late season applications are recommended.

4. *Conclusions*

Overall, the Formol method can provide a very useful index of the nutritional status of a juice or must. The simplicity of this procedure and its general ability to correctly describe the amount of assimilable nitrogen make it ideal for use in a winery production laboratory.

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Figure 1. Environmental and Processing Factors Influencing Viability and Fermentative Performance of Wine Yeasts.

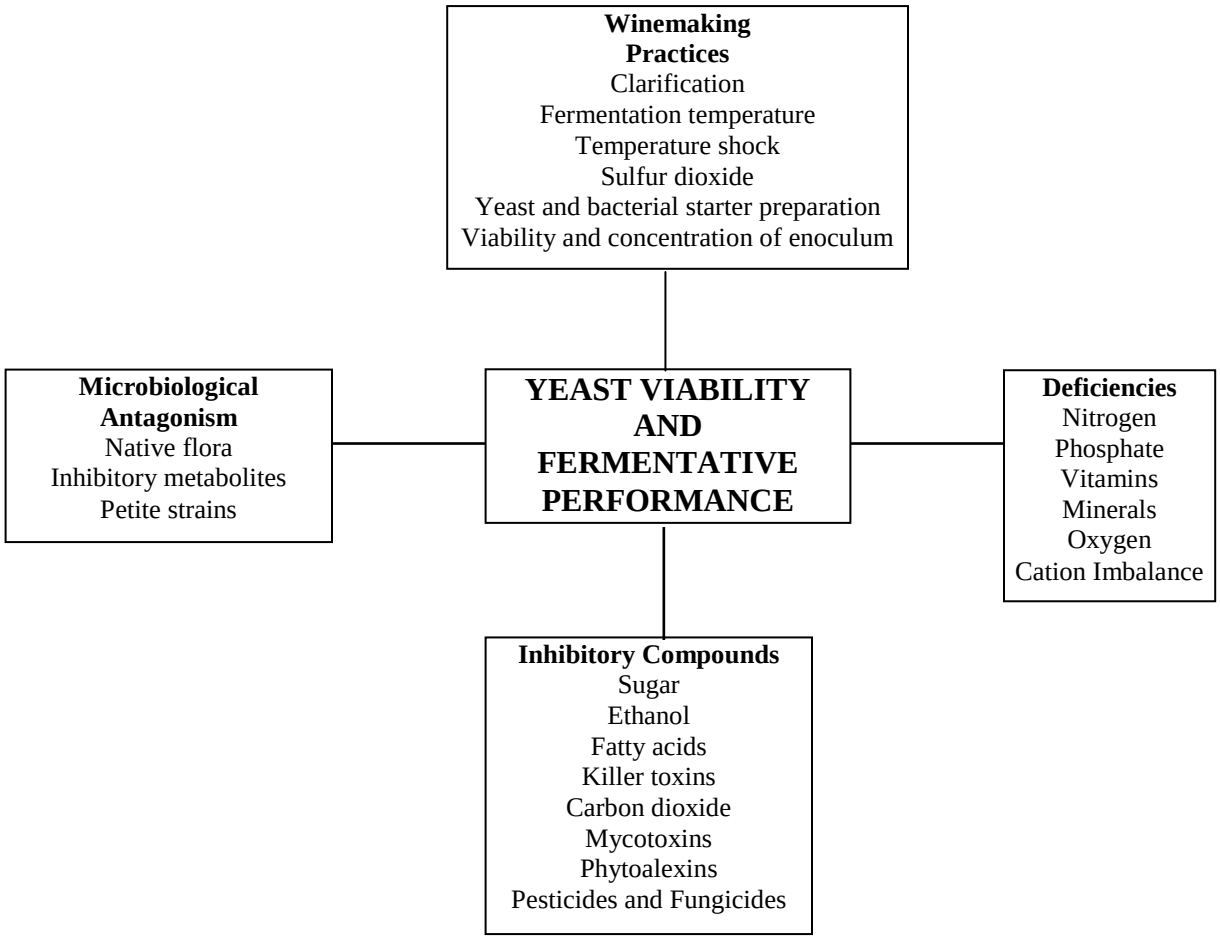
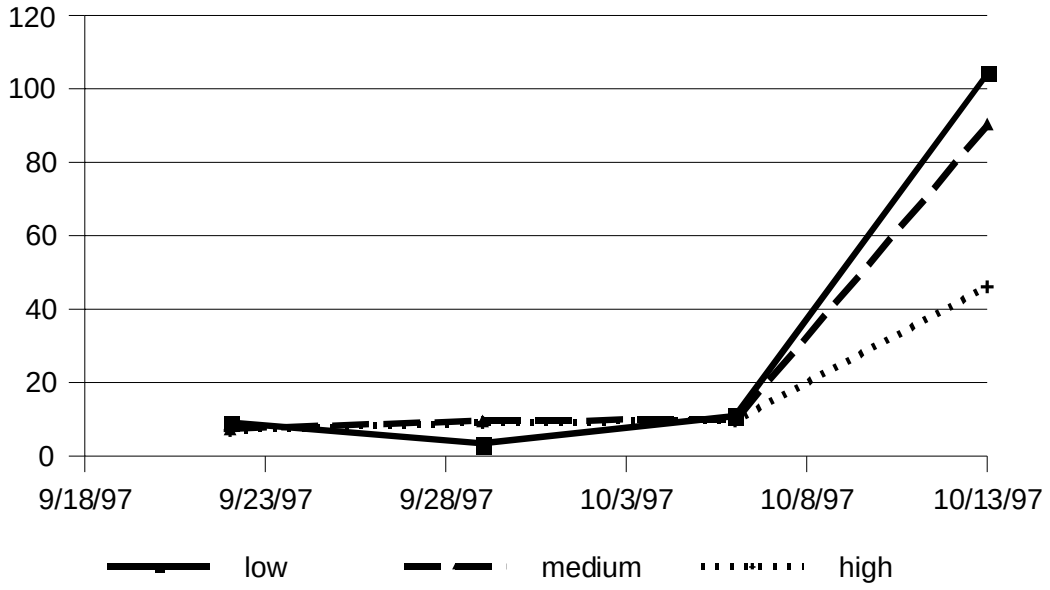
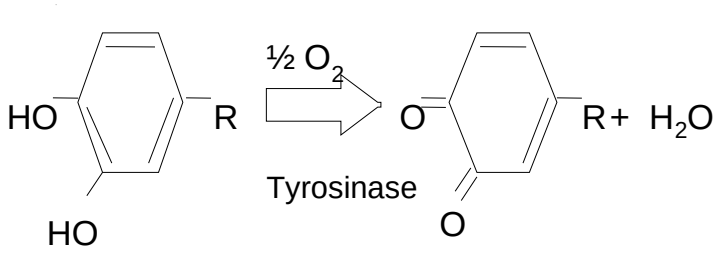


Figure 2. *Alpha*-amino nitrogen (mM) of Cabernet Sauvignon grapes cluster thinned to low (2.6 kg/vine), medium (4.9 kg/vine) and high (5.3 kg/vine) crop level in 1997.



Source: de Bordenave and Zoecklein. 1999.

Figure 3. EnzymaticOxidation of Nonfalvonoid Phenols.



(Colorless) (Brown)

Source: Zoecklein *et al.*, 1995.

Table 1: Survey results from 1993 and 1994. Mean content of free ammonia and free amino nitrogen (less free ammonia).

	Free Ammonia (mg/L)		**Free Amino Nitrogen (mg/L)**	
Year:	**1993**	**1994**	**1993**	**1994**
Cultivar				
Cayuga White	68	32	74	197
Chardonnay	46	55	151	177
Riesling	52	56	102	123
Seyval Blanc	19	14	82	156
Pinot Noir	52	88	135	116
Cabernet Sauvignon	49	69	74	142
Mean (all cultivars)	**48**	**52**	**103**	**152**

Source: Henick-Kling *et al*. (1996)