Virginia Polytechnic Institute and State University
Proposal Cover Sheet

PROPOSAL INFORMATION

Virginia Winegrowers Advisory Board
Sponsor

Solicitation No.

Nitrogen Status Of Plant Material And Juice By Arginine Titration
Proposal Title

$16,084.00	7/1/04	6/30/05	☒ New or
Amount	**Begin Date**	**End Date**	☐ Renewal of

PRINCIPAL INVESTIGATOR(S)

Bruce W. Zoecklein		
Principal Investigator	Co-Principal Investigator	Co-Principal Investigator
Bruce W Zoecklein		
Signature	Signature	Signature
231-5325		
Phone	Phone	Phone
bzoeckle@vt.edu		
Email	Email	Email
Professor		
PI Title	Co-PI Title	Co-PI Title
Food Science and Technology		
Department / Center	Department / Center	Department / Center

INSTITUTIONAL INFORMATION

SEND NOTICE OF AWARD TO:
Office of Sponsored Programs
460 Turner Street, Suite 306
Box 209
Blacksburg, Va 24060
Phone (540) 231-5283 Fax (540) 231-3599

WWW.OSP.VT.EDU
Federal Employer No. 54-6001805
Ninth Congressional District
Manufacturer or Cage Code: 4B976
DUNS No. 003137015
Make checks payable to: Treasurer of Virginia Tech

VIRGINIA TECH ENDORSEMENT

	3-9-04	04-1744-03
David W. Richardson	**Date**	**VT Proposal No.**
Director of Sponsored Programs		

RESEARCH PROPOSAL

A. TITLE: Nitrogen Status of Plant Material and Juice by Arginine Titration

B. DATE: July 1, 2004 to June 30, 2005

C. DURATION: One year for this continuing project

D. OBJECTIVES: The objectives for this year's request are to:

1) Develop a simple titration procedure for the quantification of both plant tissue and juice arginine concentrations, that is, to develop an AGROFORMOL test.

Previous studies, under the VAWAB grant titled "Nitrogen Status of Grape Juice as an Indicator of Wine Quality," have related grape juice nutritional (nitrogen) status, as determined by several analytical methodologies, to vineyard practices and wine quality. This research has allowed the PI and his colleagues to develop and optimize the Formol method (an analysis that every Virginia producer can use) for determining assimilable nitrogen levels, and determining grape nitrogen levels related to vineyard location and management practices. The objectives of this research were to:

- Evaluate the relationship between vineyard management and total assimilable nitrogen levels in grapes.
- Evaluate the relationship between vineyard management and proline/arginine ratios.
- Evaluate the relationship between proline/arginine ratios and the results from various analytical methodologies (especially the Formol titration).
- Evaluate the relationship between total nitrogen levels, proline/arginine ratio, and the ratio of fusel oils to esters, and wine quality.

Each of the above objectives has been met.

E. JUSTIFICATION / PRACTICAL IMPORTANCE: A simple, in-house test, which could easily measure both plant and juice arginine, would allow for a greater link between plant nitrogen nutrition, juice nitrogen, and ultimate wine quality.

The addition of nitrogen to the vineyard should be done with an understanding of the impact on wine quality, not simply on plant growth. Winemakers must seek to maximize production and quality, especially as international wine marketing becomes more competitive. It is essential for winemakers to have valid analytical procedures, as well as knowledge of how and when to apply these procedures, to better monitor the production of wines and help assure

maximum wine quality. With more than 35,000 new acres of California vines producing for the 2002 harvest season, competitive pressures will continue. If the Virginia industry expects to increase market share, increased quality and reduced cost of production are required.

Yeast nutrition and nitrogen deficiencies are areas of high concern. Stuck and/or sluggish fermentations can produce substantially inferior wines, in addition to compromising processing efficiency. It is also necessary to relate grape nitrogen status to specific vineyard practices and grape varieties. Poor nutrition can have dramatic sensory effects on fermented wines including:

- Stuck or protracted fermentations, which may further cause:
- The production of excessive acetic acid
- The production of excessive aldehyde
- The production of excessive fusel oils
- The limited production of esters
- Oxidative degradation

A low concentration of assimilable nitrogen, therefore, can cause fermentation sticking or, more commonly, a reduction in the floral intensity of wines. This loss of floral intensity is the result of the change in the ratio of esters to fusel alcohol. Esters are those compounds which can contribute to the floral, wine-like notes in wines. Fusel oils, on the other hand, are rather harsh and unpleasant when isolated individually. Collectively, fusel oils may contribute to complexity, if their total concentration is below 300 mg/L. Excessive addition of nitrogen in the vineyard can have an equally negative impact on wine quality. Above about 300 mg/L, there is a loss of floral character, either directly, or as a result of higher fermentation rates and increased biomass. Therefore, nitrogen application to the vineyard must be done with an understanding of the effect on wine quality. Currently, viticulturists determine the N status of their vines by sampling plant materials, sending those off to a testing lab and, after much time delay, following recommendations which are made based upon plant growth, not wine or wine quality. A simple, titration test on plant material, which could be done at the winery, would provide an opportunity to link plant tissue nitrogen status with the nitrogen status of the fruit.

Grape nitrogen also appears to be related to a sensory phenomenon known as untypical or atypical aging. Wines with this taint lose their varietal character very early, and take on atypical aromas/flavors, which have been described as naphthalene (moth balls), dirty dish rag, and wet towel. Since it was first reported, it has been identified

in wine regions of Europe, the Pacific Northwest, California, and New York. We have confirmed the presence of this in Virginia, although the incidence appears to be limited. This sensory problem has been linked to nitrogen metabolism in the vine. Aminoacetophenone and two other compounds (indole and methylindole) related to the metabolism of the amino acid tryptophan, are believed to contribute to this taint. Under certain growing conditions, the grape may accumulate excessive concentrations of these compounds in the bound, glycosidic form. These bound components may later be hydrolyzed, or broken, releasing the free odor-active volatiles and resulting in the taint. The problem appears to be related to insufficient assimilable nitrogen in the plant, but cannot simply be solved by the addition of nitrogen fertilizer in the vineyard or addition of nitrogen to the fermenter. The incidence of UTA in Virginia for the rainy 2003 season will likely be relatively low. However, seasonal variation could result in increased incidence. I have posted on my website a simple screening test that can be used to evaluate a wine's ATA potential.

Excessive amounts of nitrogen added to the vineyard can increase the arginine concentration in juice and wines, and can impact the formation of ethyl carbamate. Ethyl carbamate, or urethane, is a carcinogen that occurs naturally in fermented foods, including wine, as a result of the fermentative and assimilative activities of microorganisms. Toxicity studies are being completed by the U.S. Food and Drug Administration, prior to recommending maximum acceptable levels (R. Gahagan, 2002, personal communication). At present, the U.S. wine industry has established a voluntary target level of <15 μg/L (ppb) for table wines, and <60 μg/L for dessert wines. Because of the possible production of ethyl carbamate, it is essential that industry members understand optimum levels of fermentable nitrogen required for successful fermentation.

F. BACKGROUND: Nitrogen compounds in grapes play important roles as nutrients for microorganisms involved in the winemaking process (7). Nitrogen is taken up by the vine roots as nitrate. It is reduced by the nitrate reductase system to ammonia, transported, and stored as amino acids (23). Compared with fermentable carbon generally present in grapes at >20% (w/v), total nitrogen levels range from 0.006 to 0.24%, of which only 0.0021-0.08% is biologically available to fermenting yeasts (8). Thus, nitrogen can be an important growth-limiting constraint for yeasts.

The total nitrogen content of juice and wine is made up of protein and nonprotein fractions. Protein nitrogen comprises 1-13% of the total N (8,27), whereas polypeptides may account for more than 21%. Since *Saccharomyces*

sp. lacks both the extracellular proteases and transport enzymes necessary for protein incorporation (4), the protein fraction does not play a significant nutritional role. The nonprotein fraction of total nitrogen in juice musts includes NH_4^+ and amino acids. In grapes, NH_4^+ ranges from near 30 to more than 400 mg/L (1,2,3,11), whereas in wine, levels of less than 50 mg/L have been reported (16).

Numerous studies have demonstrated the priority of NH_4^+ uptake by yeasts, relative to amino acids. Jiranek et al. (15) and Monk et al. (17) reported NH_4^+ as not only incorporated preferentially to alpha-amino acids, but that it also alters the established pattern of amino acid uptake.

All of the 20 commonly-occurring amino acids are found in grapes and wine. Their total concentration ranges from 0.4 to 6.5 g/L (20). Of these, only the free alpha-amino acid (FAN) fraction is directly assimilable by yeasts. This fraction includes arginine, serine, threonine, and alpha-amino butyric, aspartic, and glutamic acids. Collectively, this group comprises 35-40% of the total N, and 75-85% of the total amino acids (20). Arginine is typically present at levels ranging from five to ten times that of the other amino acids, and represents 30-50% of the total nitrogen utilization (15). Thus, a simple analysis for arginine may be an effective means of gauging both the nitrogen status of the plant, and the ability of grape juice to ferment properly.

Vineyard management has a direct influence on FAN, including the arginine component (4), and fermentation problems are often vineyard-specific. Nitrogen deficiency in apparently healthy grapes can be severe, and has been demonstrated in Virginia. Drought, grapevine nutrient deficiencies, high incidences of fungal degradation, and level of fruit maturity, all influence must nitrogen. 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. Therefore, it is important to look at vineyard management at various sites, and evaluate methodologies of nitrogen availability prediction.

Minimum levels of FAN required for successful completion of alcoholic fermentation range from 120 to 140 mg/L, for musts with sugar concentrations of 160-240 g/L (26,27). FAN levels of 400-500 mg/L or greater are required for maximum fermentation rate (13,14).

Several studies have highlighted the importance of nitrogen nutrition for successful fermentations (1,6,7,8,11,13,16,18,19,20,21,22,26,27). Successful management of nitrogen deficiency requires that the winemaker identify the potential for problems, early in the winemaking process. Routine and easily performed estimates of assimilable nitrogen (FAN + NH_4^+) during juice and must processing would be a valuable tool for the winemaker. Historically, several analytical methods have been proposed to measure total nitrogen. These have included ninhydrin (16) and the trinitrobenzene sulfonic (TNBS) method described by Crowell et al. (5). Utilization of TNBS has largely been discontinued, because of difficulties in obtaining the chemical, as well as waste management issues. Further, these methods yield erroneously high results, due to inclusion of variable concentrations of protein and peptide nitrogen, and proline and other amino acids which are not readily incorporated by wine yeast.

The Formol titration is a simple and rapid method for determination of the quantity of assimilable nitrogen in juice (9,10,24,25). The PI and his colleagues have demonstrated that the Formol method can provide a very useful index of the nutritional status of a juice or must, that every winery can use. 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. Work by the author and his colleagues (10,11,28) demonstrated that Formol titrations of known concentrations of seven alpha-amino acids (alanine, arginine, serine, threonine, and alpha-amino butyric, aspartic, and glutamic acids) and proline showed quantitative recoveries from 90 to 120% for the former, and an approximately 17 to 33% recovery for proline. The percentage recovery appears to increase with the absolute amount of proline present. Similarly, Formol titrations of known concentrations of ammonium chloride solutions (at approximately 50 and 100 mg/L nitrogen) also exhibited quantitative recoveries.

Formol titrations of known mixtures of the eight amino acids mentioned above (10) also showed nearly quantitative recoveries when the titration factors (percentages recovered) for proline and arginine were considered. Our research has demonstrated that the Formol method 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 (approximately 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, the positive and negative errors in the titration essentially balance out.

Vineyard management practices influence the arginine concentration and, therefore, need to be evaluated. Samples collected under specific growing conditions from various vineyards and regions of the state and country will help provide this information.

RESULTS TO DATE: The practical significance of this research to date has been conveyed to the industry in the form of the *Vintner's Corner* newsjournals, the *Enology Notes* electronic communications, three analytical short courses, and two pre-harvest workshops.

The Formol assay has been evaluated and modified to enhance both precision and accuracy. The method was also scaled down to allow for increased efficiency. The PI and his colleagues have written two book chapters on nitrogen status and this analysis method (10,28), and one refereed publication. Three oral presentations have been given at annual meetings of the American Society for Enology & Viticulture.

Twenty-eight small lot fermentations (7 yeasts x 4 replications each) have been carried out on juice samples from selected vineyards to gain an understanding of the impact of N components on wine volatiles. Seven yeast strains were used at different levels of arginine in the must N. Wines have been analyzed for total nitrogen, ammonia, arginine, amino acids, and UTA metabolites, pre- and post-fermentation. Analytical procedures for aroma volatiles are those described by Whiton and Zoecklein (26) and Zoecklein et al. (28). Sensory analysis will be conducted, using duo-trio difference testing as described by Zoecklein et al. (29), at California State University, Fresno, in the spring of 2004.

A method for measuring volatile nitrogen components, such as methoxypyrazines (odorous N compounds responsible for the herbaceous character in some wines) has been developed, presented orally at the annual meeting of the American Society for Enology and Viticulture, and published in the *American Journal of Enology and Viticulture* (12).

G. PROCEDURES:

1) A standard addition procedure will be used to determine the quantitative recovery of arginine via Formol titration, in both model solutions and grape juice.

2) Methods for the isolation of arginine from juice and plant material (leaf petioles) will be evaluated. This will mainly involve cation exchange. The arginine concentration, along with the other FAN amino acids, will be quantified by HPLC and compared with spectrophotometric methods.

3) Juice and plant material will be titrated using the Formol procedure, and the results will be compared with both HPLC and spectrophotometric methods.

4) Plant materials and juice from 12 vineyards in Virginia and eight in California will be evaluated using the proposed AGROFORMOL procedure, and the results compared with HPLC and spectrophotometric analyses of amino acids.

H. PERSONNEL AND FACILITIES: The principal analysis for this project will occur in the Enology-Grape Chemistry Laboratory at Virginia Tech. This project requires funding for the Laboratory Technical Assistant. Vineyards in Virginia, and in the San Luis Obispo and Fresno areas of California, will be utilized.

I. OTHER ENTITIES: This research will be conducted by Virginia Tech, California State University, Fresno, and California Polytechnic State University, San Luis Obispo. Dr. Bruce Zoecklein, Department of Food Science and Technology, Virginia Tech, will serve as Principal Investigator. Dr. B. H. Gump, Cooperator, Department of Chemistry, CSUF, will help oversee analytical methodology and analyses. Professor K. C. Fugelsang, Cooperator, Department of Viticulture & Enology, CSUF, will help oversee the collection of plant tissue and juice samples. Dr. W. K. Patterson, Cooperator, Viticulturist, CSUSLO, will oversee the collection of coastal grape juice and plant tissue samples, and correlation with vineyard practices.

J. SOURCE OF OTHER FUNDS: Support funds for the previous phases of this research (completed) have been received from the California Agricultural Technology Institute ($10,000), the American Vineyard Foundation ($10,000), and ARI (Agricultural Research Initiative) ($47,000).

K. BUDGET

Reagents and supplies	$ 7,500
Wage support	8,000
Fringe (7.3%)	584
Total	$16,084

Submitted by:

Bruce W. Zoecklein
Professor of Food Science and Enology Specialist
Department of Food Science and Technology
Virginia Tech
Blacksburg, Virginia 24061

LITERATURE CITED:

1. Agenbach, W.A. (1977) A study of must nitrogen content in relation to incomplete fermentations, yeast production and fermentation activity in Proc. South African Soc. for Enol. Vitic. (Cape Town), South African Society for Enology and Viticulture, Stellenbosch, pp. 66-88.
2. Bely, M., Sablayrolles, J.-M., and Barre, P. (1990) Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferm. Bioeng. 70(4), 246-252.
3. Bely, M., Sablayrolles, J.-M., and Barre, P. (1991) Automatic detection and correction of assimilable nitrogen deficiencies during alcoholic fermentation in enological conditions in Proceedings of the International Symposium on Nitrogen in Grapes and Wine (J. Rantz, Ed.), American Society for Enology and Viticulture, Davis, California, pp. 211-214.
4. Bisson, L.F. (1996) Yeast and biochemistry of ethanol formation in Principles and Practices of Winemaking (R.B. Boulton, V.L. Singleton, L.F. Bisson and R.E. Kunkee, Eds.), Chapman & Hall, New York, p. 140.
5. Crowell, E.A., Ough, C.S., and Bakalinsky, A. (1985) Determination of alpha-amino nitrogen in musts and wines by TNBS methods. Am. J. Enol. Vitic. 36, 175-177.
6. Dittrich, H.H. (1987) Mikrobiologie des Weines. Handbuch der Lebens-mitteltechnologie, 2nd ed., Ulmer, Stuttgart.
7. Dukes, B.C., and Butzke, C.E. (1998) Rapid determination of primary amino acids in grape juice using an o-phthaldialdehyde/N-acetyl-L-cysteine spectrophotometric assay. Am. J. Enol. Vitic. 49, 125-134.
8. Fugelsang, K.C. (1996) Wine Microbiology. Chapman and Hall, New York.
9. Giannessi, P., and Matta, M. (1978) Azoto Amminico in Trattato di Scienzae Technicanologica, Vol. I (AEB Brescia, Ed.), Analisi e Controllo dei mosti e dei Vini, pp. 87-88.
10. Gump, B.H., Zoecklein, B.W. , and Fugelsang, K.C. (1999) Prediction of prefermentation nutritional status of grape juice - The Formol method in Food Microbial Protocols in Methods in Molecular Biology (J.M. Walker, Ed.), Hanna Press, Palo Alto, California.
11. Gump, B.H., Zoecklein, B.W., Fugelsang, K.C., and Giannini, J. (1999) The Formol titration for predicting nitrogen nutritional status, presented at the American Society for Enology & Viticulture - Eastern Section meeting, St. Louis, Missouri, July 16th.
12. Hartmann, P.J., McNair, H.M., and Zoecklein, B.W. (2002) Measurement of 3-alkyl-2-methoxypyrazine by headspace solid-phase microextraction in spiked model wines. Am. J. Enol. Vitic. 53, 285-288.
13. Henick-Kling, T., Edinger, W.D., and Larsson-Kovach, I.-M. (1996) Survey of available nitrogen for yeast growth in New York grape musts. Die Wein-Wiss. 51(3), 169-174.
14. Henschke, P.A., and Jiranek, V. (1993) Metabolism of nitrogen compounds in Wine Microbiology and Biotechnology (G.H. Fleet, Ed.), Harwood Academic Pubs., Australia, pp.27-54.
15. Jiranek, V., Langridge, P., and Henschke, P.A. (1990) Nitrogen requirement of yeast during wine fermentation in Proceedings of the Seventh Australian Wine Industry Technical Conference (P.J. Williams, D.M. Davidson, and T.H. Lee, Eds.), Australian Industrial Publishers, Adelaide, S.A., pp. 166-171.
16. Lie, S. (1973) The EBC-Ninhydrin method for determination of free alpha-amino nitrogen (FAN). J. Inst. Brew. 79, 37-41.
17. Monk, P.R., Hook, D., and Freeman, B.M. (1987) Amino acid metabolism by yeast in Proceedings of the Sixth Australian Wine Industry Technical Conference (T.H. Lee, Ed.), Australian Industrial Publishers, Adelaide, SA, pp. 129-133.
18. Ough, C.S. (1969) Substances extracted during skin contact with white must. I. General wine composition and quality changes with contact time. Am. J. Enol. Vitic. 20, 93-100.
19. Rapp, A., and Versini, G. (1991) Influence of nitrogen compounds in grapes on aroma compounds in wine in Proceedings of the International Symposium on Nitrogen in Grapes and Wine (J. Rantz, Ed.), American Society for Enology and Viticulture, Davis, California, pp. 156-164.
20. Sablayrolles, J.-M. (1992) Importance de l'azote assimilable de l'oxygene sur le deroulement de la fermentation alcoolique. Biol. Oggi 6, 155-160.
21. Sablayrolles, J.-M. (1996) Sluggish and stuck fermentations. Effectiveness of ammonium nitrogen and oxygen additions. Die Wein-Wiss. 51(3), 147-151.

22. Sablayrolles, J.-M., and Dubois, C. (1996) Effectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck fermentations. J. Ferm. Bioeng. 92, 377-381.
23. Schulze, U., and Liden, G. (1996) Physiological effects of nitrogen starvation in an anaerobic batch culture of *Saccharomyces cerevisiae*. Microbiol. 142, 2299-2310.
24. Sponholz, W.R. (1991) Nitrogen compounds in grapes, must, and wine in International Symposium on Nitrogen in Grapes and Wine (J. Rantz, Ed.), American Society for Enology and Viticulture, Davis, California, pp.67-77.
25. Taylor, W.H. (1957) Formol titration: an evaluation of its various modifications. Analyst 82, 488-498.
26. Whiton, R.S., and Zoecklein, B.W. (2000) Optimization of headspace solid-phase microextraction for the analysis of wine aroma compounds. Am. J. Enol. Vitic. 51, 379-382.
27. Wurdig, G., and Woller, R. (1989) Chemie des Weines. Handbuch der Lebensmittel-technologie. Ulmer, Stuttgart.
28. Zoecklein, B.W., Gump, B.H., and Fugelsang, K.C. (1999) Nutritional status of grape juice in Food Microbial Protocols in Methods in Molecular Biology (J.M. Walker, Ed.), Hanna Press, Palo Alto, California.
29. Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., and Nury, F.S. (1995) Wine Analysis and Production. Chapman and Hall Publishers, New York.

BRUCE W. ZOECKLEIN
Associate Professor
BRIEF PROFESSIONAL BIOGRAPHY

I. Résumé

Educational Background:

Ph.D., Food Science and Technology, Virginia Tech. 1995.

M.S., Horticulture, Virginia Tech. 1993.

B.S., Microbiology, California State University-San Diego. 1972.

Previous Experience:

Associate Professor, Enology Specialist, Department of Food Science and Technology, Virginia Tech. 1999.

Assistant Professor, Enology Specialist, Department of Food Science and Technology, Virginia Tech. 1995.

Research Associate, Enology Specialist, Department of Food Science, Virginia Tech. 1994-1995.

Research Associate, Enology Specialist, Department of Horticulture, Virginia Tech. 1985-1994.

Extension Specialist-Enology, Department of Horticulture, University of Missouri. 1980-1985.

Research Specialist-Enology, Viticulture Research Station, California State University, Fresno. 1978-1980.

Instructor, Department of Food Science-Enology, California State University, Fresno. 1977-1980.

Production Manager, Oliver Wine Company, Bloomington, Indiana. 1975-1977.

Winemaker, A. Perelli-Minetti and Sons Winery (California Wine Association), Delano, California. 1973-1975.

Winemaker, Pleasant Products Food Corporation, San Diego, California. 1971-1973.

Honors and Awards:

Virginia Tech Gamma Sigma Delta Teaching Merit Award. 2000.

Napa Valley Wine Council Research Award, shared with K.C. Fugelsang. 1999

Virginia Tech Gamma Sigma Delta Extension Merit Award. 1999.

Virginia Tech Alumni Extension Excellence Award. 1997. The highest honor given by Virginia Tech for excellence in extension programs.