AN ABSTRACT OF THE THESIS OF

Anna Louise Specht for the degree of Master of Science in Food Science and Technology presented on May 25, 2000. Title: Evaluation of the Effects of Nutrient Supplementation on Low Nitrogen Chardonnay.

Abstract approved-

Barney T. Watson

Low nitrogen Chardonnay juice from an Oregon winery was fermented with a series of nutrient supplements, including diammonium phosphate (DAP), Fermaid K, Superfood, yeast extract, yeast hulls and thiamine. These treatments were evaluated for their contribution to the yeast assimilable nitrogen content (YANC) of the juice, which consists of the ammonium ion and α-amino acid content by NOPA (nitrogen by *o*-phthaldialdehyde). The fermentation characteristics and the nutritional and chemical status of the finished wines were compared.

Diammonium phosphate added 25 mg N/L from ammonia for every pound/1000 gallons (12.5 g/hL) used. Commercial yeast nutrient preparations were found to contribute around 10 mg N/L from ammonium ion per pound/1000 gallons (12.5 g/hL). NOPA and HPLC

analysis of Superfood and Fermaid K indicated that they contributed only a small amount of amino acids. NOPA analysis of PL-50 yeast extract indicated that it may have added some nitrogen from amino acids.

Nitrogenous compounds were taken up within the first few days of fermentation. Ammonium ion was depleted very quickly and did not reappear later in fermentation. Alpha-amino acids reached a low within approximately ten days of fermentation, then leveled out or increased towards the end of fermentation. Treatments containing high amounts of ammonia were observed to produce the most vigorous fermentations. These treatments were found to have the highest apparent levels of amino acids at the end of fermentation. The Control treatment, which had the lowest YANC content, appeared to utilize proline during fermentation.

Sensory analysis was not conducted on the finished wines due to replication differences in reduced sulfur aroma found in preliminary screening. GC-MS analysis showed that the higher nitrogen treatments had levels of reduced sulfide compounds that were above sensory threshold, whereas moderate nitrogen treatments did not.

Ethyl carbamate was not found in any of the finished wines.

These data suggest that moderate levels of supplementation had a positive effect on both fermentation and finished wine quality. Higher levels of nutrient addition were effective at increasing fermentation rates but negatively affected the sulfide profile of finished wines.

EVALUATION OF THE EFFECTS OF NUTRIENT SUPPLEMENTATION ON LOW NITROGEN CHARDONNAY

by

Anna Louise Specht

A THESIS

Submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented May 25, 2000

Commencement June 2001

Master of Science thesis of Anna Louise Specht presented on May 25, 2000

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Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University Libraries. My signature below authorizes the release of my thesis to any reader upon request.

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ACKNOWLEDGMENTS

Several kind people helped with processing and analysis. I am grateful to Sara Spayd and David Mee of Washington State University, Prosser, WA, who performed sulfides analysis, Gordon Burns of ETS Labs for performing ethyl carbamate and amino acid analysis, Steve Price for help with the preliminary sensory screening of the wines, Hsiao-Ping Chen for her help in the lab, and Kate Wall for helping me siphon many many small bottles of wine.

I would like to thank my parents, David and Caroline Specht, for being patient and supportive. Thank you to Steve for waiting so long. Thank you to my friends at OSU for keeping me constantly entertained: Laurie, Matt, Uri, Jeff, Brian, Nate, Jessica, Kate, Jason, Dawn, Bryan, Keith, Lotika & Viv, and Tina.

Finally, I would like to thank Barney Watson for letting me tag along. I couldn't have had more fun learning about wine.

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for Boompa

I miss you

EVALUATION OF THE EFFECTS OF NUTRIENT SUPPLEMENTATION ON LOW NITROGEN CHARDONNAY

1. INTRODUCTION

Stuck or sluggish fermentations occur when yeasts stop converting sugar to ethanol at an unacceptably high level of residual sugar. In wine, this condition contributes to several types of quality degradation. Wine that is not fermented to dryness is at a much higher risk of infection by spoilage microorganisms, whose metabolites can cause "off"-flavors and aromas. In addition, there is a complex relationship between the alteration of yeast nitrogen metabolism and the overproduction of sulfide compounds, which can contribute negatively to the sensory profile of the wine. Finally, fermenting wine is protected from oxidation by a blanket of CO_2, which can be lost in sluggish fermentations. If the finishing operations of racking and topping are delayed, extended exposure to air can leave the wine unprotected from oxidation (Alexandre and Charpentier, 1998).

Several factors can lead to stuck fermentation, including deficiencies in assimilable nitrogen (Salmon, 1989; Bely *et al.* 1990 a & b; Jiranek *et al.* 1991; Kunkee, 1991; Sponholz, 1991), vitamins (Ough *et al.* 1989) and trace elements (Montiero and Bisson, 1991; Alexandre and Charpentier, 1998); inhibition by sugar (Ingledew and Kunkee, 1985), ethanol (van Uden, 1989),

pesticide residues (Lemperle, 1988) and SO_2 (Lehmann, 1987); clarification (Vos and Gray, 1979; Houtman *et al.* 1980), temperature (Amerine *et al.* 1980) and inadequate agitation (Ingledew and Kunkee, 1985). Nutrient deficiency, particularly nitrogen deficiency, is the focus of this study.

Nitrogenous compounds are precursors to structural proteins (van der Rest *et al.* 1995), enzymes and nucleic acids (Bisson, 1991) that are necessary for rapid yeast growth and division. They also affect the rate of sugar transport and, therefore, metabolic rate (Busturia and Lagunas, 1986).

Yeasts are only able to use so-called "fermentable" sources of nitrogen; that is, primary amines, ammonium ion, urea, small peptides, purines and pyrimidines (except thiamine) (Boulton *et al.* 1996). For the purposes of routine analysis, the amount of fermentable nitrogen in the juice, known as "yeast assimilable nitrogenous compounds" (YANC), is the sum of the ammonium ion and primary amine content.

The YANC content of grape juice is influenced by fruit maturity, variety, vineyard location, fertilization and irrigation regimes, vineyard management practices and fungal depletion by *Botrytis cinerea*, *Aspergillus*, or other forms of rot (Henschke and Jiranek, 1993). When juice nitrogen is low, winemakers often add commercially available preparations that provide ammonia, amino acids and other fermentation-enhancing compounds to the must (Munoz and Ingledew, 1989a and b; Ough *et al.* 1989; Oura and Suomalainen, 1978; Montiero and Bisson, 1992a and b,; Ingledew *et al.* 1986; O'Connor-Cox *et al.*, 1991; Ingledew, 1995). Such

products range from diammonium phosphate (DAP), an inexpensive and common source of ammonium ion and phosphate, to commercial preparations such as "Superfood" and "Fermaid." These products include a wide assortment of vitamins, minerals, yeast extracts and yeast hulls in addition to nitrogen-containing components. Whether these products are significant contributors of primary amines has not been established; neither has it been determined how the addition of these products affects the fermentation performance, chemical composition, and sensory attributes of the finished wine.

This trial compares fermentation characteristics and chemical components of low nitrogen Chardonnay fermented after supplementation by a series of nutrient preparations. These characteristics include fermentation performance, residual nitrogen content, amino acid composition throughout fermentation, formation of sulfide compounds and ethyl carbamate, and the sensory characteristics of the finished wine.

2. LITERATURE REVIEW

2.1 INTRODUCTION

During alcoholic fermentation, the wine yeast *Saccharomyces cerevisiae* uses nitrogen compounds in many metabolic processes. Yeasts assimilate nitrogen in the form of ammonium ion and α-amino acids, which are used in the biosynthesis of enzymes, structural proteins and nucleic acids (Boulton *et al.*, 1996). Vitamins such as thiamine, calcium pantothenate and biotin are cofactors needed nitrogen and sugar metabolism (Ough *et al.*, 1989; Castor, 1952).

Occasionally, the amount of YANC in grape juice is low enough to limit yeast growth, leading to "sluggish" or "stuck" fermentations (Ough, 1964; Bell *et al.* 1979; Ingledew and Kunkee, 1985; Ingledew *et al.* 1986). In a stuck fermentation, the yeast population is unable to convert all of the available carbohydrate into ethanol. The consequences of sticking can range from minor, if wines can be blended to obtain the desired level of residual sugar, to severe in a case where fermentation cannot be restarted. Sulfide compounds, producing rotten egg-, garlic- or natural gas-like odors, are reportedly associated with nitrogen or vitamin deficiency (Vos and Gray, 1979; Wainwright, 1970; Okuda and Oemura, 1965). Extended fermentations can create oxidation problems due to delayed racking and commercial loss from inefficient utilization of tank space (Alexandre and

Charpentier, 1998). In severe cases, the wine is totally unusable and must be discarded. The problem of incomplete fermentation has been cause for increasing concern among winemakers, who want to reduce the risks associated with the fermentation of nitrogen-poor musts.

There have been several approaches to solving the problem of nitrogen deficiency. Certain vineyard blocks often produce nitrogen poor fruit year after year (Watson *et al.*, 2000a). Vineyard management practices, such as nitrogen fertilization, tilling, and irrigation have been aimed at reducing nitrogen deficiency at harvest (see 2.4.1). There has been an effort from the biochemical side to design a yeast that is capable of efficiently utilizing available nitrogen from proline and arginine (Salmon and Barre, 1998); the use of this yeast with low nitrogen musts could prevent stuck fermentation. A practical and convenient solution has been the addition of nutritional supplements at the onset of fermentation to raise the amount of YANC to an appropriate level (Ingledew *et al.*, 1986; Munoz and Ingledew, 1989 a and b; Ough *et al.*, 1989; O'Connor-Cox *et al.*, 1991; Montiero and Bisson, 1992 a and b; Ingledew, 1995). Such supplements include diammonium phosphate (DAP), yeast hulls, yeast extract, vitamins and a number of commercial preparations which combine all of the above in proprietary mixtures.

The timing of supplement additions is a matter of some controversy. Most winemakers add nutrients to juice or must prior to inoculation with the yeast, although some promote addition of nitrogenous compounds

periodically throughout fermentation (Manginot *et al.*, 1997). The recommended yeast assimilable nitrogen content (YANC) in the juice ranges from 140 mg N/L (ppm) to 500 mg N/L (Butzke, 1998). However, the legal limit of most of these supplements in the U.S. is eight pounds per 1000 gallons (100 g/hL) (CFR, 24.246, 1991). A one-pound/1000 gallon (12.5 g/hL) addition of DAP increases the YANC of the juice by approximately 25 mg N/L; thus the maximum possible legal addition of nitrogen in the U.S. is limited to 200 mg N/L. Sources of amino acids, such as yeast extract, often contain 'stinky' components and can lead to the production of unpleasant aromas. Their use is limited to lower amounts (one-pound/1000 gallons, or 12.5 g/hL).

Supplements are a seemingly simple remedy for nitrogen deficiency. It might be tempting to add the legal limit of a supplement, regardless of juice composition, in the hopes of avoiding stuck fermentation and the development of 'off'-type sulfur odors. However, there may be several quality parameters to consider when supplementing musts with yeast nutrients. The relationship between nitrogen supplementation and sulfur metabolism is poorly defined (Jiranek *et al.*, 1995). Residual nitrogen in the finished wine could result in microbial instability, possibly leading to spoilage or promoting malolactic fermentation (Castor, 1952), which may or may not be desirable stylistically. In addition, several nitrogen compounds, including urea, arginine, and carbamoyl phosphate, are potentially precursors for the formation of ethyl carbamate, a carcinogen

found in fermented foods (Ough *et al.*, 1988; Henschke and Ough, 1991; Ingledew *et al.* 1987a). Because amino acid compounds contain aroma and flavor precursors, it is possible that adding excess nitrogen of any type can lead to sensory differences in the finished wine (Rapp and Versini, 1991; Guitart *et al.*, 1999; Herraiz and Ough, 1992; Juhász and Törley, 1985). Finally, excess supplementation is undesirable from a financial standpoint, as some supplements are more costly than others. From both economic and quality perspectives, the addition of these supplements needs evaluation.

2.2 YEAST

Stuck fermentation occurs as yeasts respond to environmental and nutritional stresses by decreasing or completely shutting off sugar uptake (Salmon, 1989; Salmon *et al.*, 1993; Bisson, 1999). To understand this process, it is necessary to consider several aspects of the growth and metabolism of the wine yeast, *Saccharomyces cerevisiae*.

2.2.1 YEAST GROWTH KINETICS

As with other microorganisms in batch culture, yeast populations exhibit a predictable growth curve with four distinct phases (Prescott *et al.*, 1996).

Lag phase During lag phase, the seeded population adjusts to the fermentation medium, taking up nutrients and preparing for growth.

Growth phase Final cell density is determined during exponential or growth phase, when yeasts replicate at a rapid rate. The demand for carbon and nitrogen compounds is highest during growth phase (Jiranek *et al.*, 1991). Nitrogenous compounds are generally depleted mid-way through exponential phase, after which cells generally undergo two more rounds of cell division (Bisson, 1993).

Stationary phase Replication and cell growth stop during stationary phase, although metabolism continues; approximately 50% of sugars are consumed in stationary phase (Bisson, 1993).

Death phase Metabolism declines during death phase, as cells begin to die off. Despite the waning health of cells under conditions of high alcohol, continued fermentation is necessary to metabolize the last of the residual sugar (Ribéreau-Gayon, 1985).

The nutritional status of the yeast population during each phase can have an effect on the nutritional status during subsequent phases. For instance, nitrogen limitation during growth phase can lead to nitrogen-depleted stationary phase cells (Bisson, 1993). Although nitrogenous compounds are no longer needed for bulk cellular reproduction during stationary phase (Jiranek *et al.*, 1991), nitrogen deficiency at this point may decrease hexose transport (Salmon *et al.*, 1993) (see section 2.2.2.1), leaving more sugar to ferment during death phase. The fermentation may finally

stick when ammoniacal reserves are depleted in death phase cells (Salmon, 1989).

2.2.1.1 GROWTH CURVE CONTROL

A number of parameters govern the shape of the yeast growth curve and as such, the kinetics of fermentation are highly variable (Bely *et al.*, 1990b). In grape juice, factors such as temperature, nutritional status, yeast type, prefermentation clarification, SO_2 addition, inoculum level, and microbial status affect the yeast population at any given time (Fleet and Heard, 1993; Dubois *et al.* 1996). Nishino *et al.* (1985) found that high osmotic pressure created by increased sugar concentration (a condition that increases the risk of stuck fermentation) delays the onset of budding in *Saccharomyces*, slowing growth rate. Metabolism during death phase may be extended in the presence of "survival factors" (see section 2.2.2), decreasing the likelihood of stuck fermentation. Growth phase performance can be related to the death phase aspect; for instance, the addition of nutrient supplements to ensure a vigorous growth phase may prolong the cell's ability to synthesize proteins during death phase, maintaining viability long enough to complete fermentation (Salmon, 1989).

Addition of nutrient supplements during lag phase (or before inoculation with yeast) ensures that nutrients will be available during the exponential phase. Adequate nutrition in exponential phase ensures that

the yeast population achieves maximum cell density. Generally, yeasts are seeded at a density of 10^4-10^6 cfu/ml and increase to 10^8-10^9 cfu/ml by stationary phase (Fleet and Heard, 1993). Cultures that do not reach this cell density can have trouble completing fermentation, as they lack the biomass to process the high levels of sugar found in grape juice (Lafon-Lafourcade *et al.*, 1979).

2.2.1.2 MODELS

Bezenger and Navarro *et al.* (1987) developed a model specifically designed to account for nitrogen addition at the onset of fermentation. This model established a relationship between CO_2 evolution and nitrogen content, from 79-266 mg N/L, and was able to accurately predict a maximum rate of CO_2 production at ~200 mg N/L. Models such as this one use pH, temperature, cell density, and nutrient status to predict fermentation kinetics. It is difficult to extrapolate the results of such studies to other conditions, however, where variables such as grape variety, vintage, and grape maturity cause wide variation in fermentation kinetics (Dubios *et al.*, 1996).

2.2.2 *NUTRIENT METABOLISM*

Carbon and nitrogen metabolism are the workhorses of yeast growth and fermentation. They generate energy as well as the macromolecular

components, such as cell wall material, enzymes and nucleotides, necessary for replication. Deficiencies in nutritional components can lead to cessation of sugar uptake and protein synthesis, resulting in stuck fermentation.

2.2.2.1 SUGAR TRANSPORT

During fermentation, *Saccharomyces cerevisiae* derives energy from the catabolism of hexose sugars, mainly glucose and fructose. These sugars are transported across the plasma membrane by permeases, which use facilitated diffusion (non-energy requiring) to import sugars into the cell (Gancedo and Serrano, 1989). Although there is an active transport (energy requiring) permease for disaccharides, sucrose is usually cleaved by an extracellular invertase before uptake (Bisson, 1993).

Sugar uptake in eukaryotes is a complicated system that has not been successfully reproduced *in vitro*. It involves multiple carriers which have different affinities for hexose substrates depending on environmental conditions (Bisson, 1993). High affinity carriers are able to recognize only one vector of approach for a sugar molecule, and thus function at a lower maximum velocity than low affinity carriers, which recognize a broader range of sugar conformations. Low affinity carriers can be repressed in high glucose concentrations due to competitive inhibition, in which several sugar molecules jam the permease and prevent translocation.

Hexose transport is the main site for rate control in glycolytic flux, and therefore fermentation rate (Salmon *et al.*, 1993). In *Saccharomyces cerevisiae*, there are up to 18 types of carriers, which are part of the *HXT* (hexose transporter) multigene family found in yeast (Bisson, 1999). The existence of so many carriers may help yeast to detect the concentration of sugar substrate (Bisson, 1993). This multitude of carriers also provides a mechanism for sensitive regulation of sugar uptake. Transport rate needs to be closely cued to glycolysis in order to prevent the build up of intracellular free glucose, which is toxic (Becker and Betz, 1972). In the event that transport needs to be slowed, yeasts are able to strategically eliminate an entire subset of transporters, rather than trying to partially reduce the transport activity of all constituent permeases (Bisson, 1999).

Glycolytic flux is also regulated by nitrogen content (Bisson, 1993). Nitrogen limitation decreases hexose transport activity (Busturia and Lagunas, 1986). Ammonium ion is an allosteric effector of several glycolytic enzymes (Sols, 1981; Rhodes *et al.*, 1986), including phosphofructokinase and pyruvate kinase.

2.2.2.2 CATABOLISM

Under fermentative conditions, less than 10% of carbon is utilized through respiration (Gancedo and Serrano, 1989). Fermentation, while not as efficient at generating ATP as respiration, has many benefits for yeast. Fermentation is fast and metabolically inexpensive when compared to

respiration (Boulton *et al.*, 1996). When sugar is not limiting, it permits rapid growth in an environment where yeasts may need to gain a competitive advantage over other microorganisms. In addition, it results in the production of alcohol, a toxic metabolite for which *S. cerevisiae* has a higher tolerance than other competing microorganisms (Boulton *et al.*, 1996).

Glycolysis converts hexose sugars to pyruvate and generates reducing power (NADH). During subsequent alcoholic fermentation, pyruvate is converted to acetaldehyde and CO_2 via pyruvate decarboxylase. This reaction, as with all α-ketoglutarate decarboxylase reactions, requires the coenzyme thiamine pyrophosphate. Acetaldehyde, the terminal electron acceptor in alcoholic fermentation, is then reduced to ethanol by alcohol dehydrogenase, regenerating NAD^+ from NADH.

2.2.2.3 ANAPLEROTIC METABOLISM

During anaerobic fermentation, *Saccharomyces cerevisiae* does not use the tricarboxylic acid cycle (TCA) to generate energy, and does not maintain the enzymatic machinery in its mitochondria that is normally associated with the TCA cycle (respiration). However, those same enzymes and others are needed for anaplerotic reactions, that is, the regeneration of TCA precursors for nucleotide and amino acid synthesis. In order to solve this problem, yeasts maintain a complement of TCA enzymes in the cytoplasm

(Boulton *et al.*, 1996). Several of these systems are sensitive to nutrient deficiency.

Anaplerotic pathways that are sensitive to nutrient deficiency include oxaloacetate and α-ketoglutarate anabolism. These molecules are transaminated to aspartate and glutamate, which are important biosynthetic precursors (Mathews and Van Holde, 1996). Oxaloacetate is generated from pyruvate by the enzyme pyruvate carboxylase, which uses biotin as a CO_2 carrier. Biotin is carboxylated in an ATP dependent reaction, after which the carboxyl group is transferred to pyruvate. The resulting oxaloacetate undergoes a transamination reaction to produce aspartate, which is a critical precursor to the purines, pyrimidines and proteins necessary for cell growth. Biotin limitation can thus lead to depression of purine nucleotide and arginine synthesis (Oura and Suomalainen, 1978).

2.2.2.4 NITROGEN METABOLISM

Yeasts are able to use several sources of nitrogen, including ammonium ion, selected primary amines, urea, and small peptides (Large, 1986). These are generally broken down into ammonia or glutamate for use in the biosynthesis of other nitrogen-containing compounds. Proline, often the most predominant amino acid found in grape juice, is generally not used under the anaerobic conditions of fermentation (Ingledew *et al.*, 1987b). Arginine, another predominant amino acid, contains four nitrogen atoms;

however, because it is degraded to proline, only three of them are generally available during fermentation. Yeasts are also able to use nitrogenous bases. Guanine and adenine can be catabolized to allantoin, which is degraded to urea (Cooper, 1980). This urea is then degraded to ammonia by urea carboxylase.

The first nitrogen compounds taken up by yeast during growth phase are used to fill intracellular amino acid pools (Cooper, 1980). The existence of these pools allows cells to take up nitrogen quickly, sequestering it in the vacuole or cytoplasm for use in protein synthesis (Boulton *et al.*, 1996). The composition of these pools reflects the composition of the medium, although glutamic acid accumulation is favored under most conditions (Watson, 1976). Although ammonia is a preferred nitrogen source, it is taken up secondarily to the amino acids used to fill intracellular pools (Jones and Pierce, 1964). Nitrogen compounds assimilated during subsequent uptake are used for growth, saving amino acid pools for use as needed (Boulton, 1996).

Before nitrogen compounds can be utilized, they must first be transported into the cell, usually via active transport through a permease protein. Permeases have general or specific affinities for amino acids, and may or may not be induced by specific amino acids (Lasko, 1981). Permeases are regulated by nitrogen so that transporter activities are low in the presence of a preferred source such as ammonium ion or glutamate (Courchesne and Magasanik, 1983). For example, the activity of the general

amino acid permease in *Saccharomyces cerevisiae* is maximal when cells are grown on a poor nitrogen source such as proline, but is almost undetectable in cultures grown on ammonium sulfate (Roon *et al.*, 1975). This phenomenon is known as "nitrogen catabolite repression" (Salmon and Barre, 1998).

Nitrogen catabolite repression occurs as a function of the products of three proteins. *GLN3* and *URE2* regulate transcription of genes for assimilatory pathways, such as the proline utilization pathway, when preferred nitrogen sources are not available (Courchesne and Magasanik, 1988). The third protein, the general amino acid permease *GAP1*, is inactivated by dephosphorylation (Stanbrough and Magasanik, 1995) during regulation by *GLN3* and *URE2*. Repression is often irreversible, as is the case with the proline specific permease *PUT4* (Courchesne and Magasanik, 1983). This can cause problems later, when the preferred nitrogen source is depleted and yeasts are incapable of utilizing the remaining non-preferred sources.

Genetic alteration is one approach to circumventing nitrogen catabolite repression. Salmon and Barre (1998) attempted to avoid the suppression of proline utilization with a *URE2* mutant that deregulated the proline utilization pathway. This improved both proline and arginine utilization, even in the presence of preferred nitrogen sources. Proline utilization in this experiment was facilitated by the addition of very small amounts of dissolved oxygen.

Enzymes for amino acid catabolism are regulated at the transcriptional level by preferred nitrogen sources as well (Magasanik, 1982). In *Saccharomyces cerevisiae*, Courchesne and Magasanik (1983) showed that arginase, a catabolic enzyme, was induced by arginine and repressed by ammonia.

2.2.2.5 SURVIVAL FACTORS

"Survival factors," usually fatty acids and sterols, serve a number of functions that prolong yeast performance during the death phase of fermentation. Saturated fatty acids found in yeast hulls can act as oxygen substitutes in poorly aerated fermentations. Munoz and Ingledew (1989a and b) found that yeast hulls, ergosterol and Tween 80 (containing oleic acid) enhanced fermentations that had poor initial aeration.

Long chain fatty acids found in yeast hulls also prolong death phase by contributing to membrane fluidity later in fermentation. High concentrations of alcohol in the medium decrease membrane flexibility and promote leakage of protons and metal ions (Boulton, 1996).

2.2.3 EVOLUTION OF SULFIDE COMPOUNDS

Sulfide compounds, including H_2S, thiols, and thioacetates, produce rotten egg-, garlic- or burnt rubber-like odors, and can be unpleasant byproducts of fermentation. Sulfur is assimilated by yeast in the form of

sulfate (SO_4^-) or sulfite (SO_3^-), which is then reduced by the sulfate reduction pathway (Rauhut, 1996) to sulfide compounds for utilization in the biosynthesis of sulfur-containing amino acids, methionine and cysteine. Under conditions of "normal" yeast nutrition, the reduction of sulfur compounds is closely matched to the demand for biosynthetic intermediates, and under tight control because of its high energetic cost (Henschke and Jiranek, 1991).

The metabolic events that lead to sulfide overproduction are poorly understood. It is thought to be due to multiple interactions that rely heavily on the total nutritional composition of the juice (Sea *et al.*, 1998). It has been observed that nitrogen supplementation can decrease sulfide odors in wine (Vos and Gray, 1979; Jiranek *et al.*, 1991 and 1995a). Vos and Gray (1979) proposed that nitrogen-deficient yeast make a protease that salvages nitrogen from extracellular proteins. Sulfide compounds would then be liberated from methionine and cysteine during proteolysis. However, the presence of such a protease has not been demonstrated. The presence of sulfides in nitrogen deficient juice could be due to a malfunction of the sulfate reduction sequence. Jiranek *et al.*, (1995a) observed several fermentations supplemented with assimilable nitrogen in which sulfides were liberated during exponential phase. The peak of sulfide formation occurred immediately after depletion of ammonium ion, but only when this occurred as cells were still multiplying. This indicated that the extent of sulfide production was controlled by the rate of the

sulfate reduction sequence, which was proportional to the rate of cell growth.

Hydrogen sulfide odor can be minimized by the addition of copper sulfate, although legal limits may prevent complete elimination of H_2S in all cases. Problems often arise when H_2S forms complex sulfides, such as disulfides, which are not removed by copper sulfate. Many of these compounds have higher sensory thresholds, and may not be detected until too late (after bottling), when they revert to a reduced form with a lower sensory threshold. This situation can make sulfide problems especially persistent (Bobet *et al.*, 1990).

2.2.4 *Evolution of ethyl carbamate*

Residual nitrogen compounds in wine, namely urea, arginine and carbamoyl phosphate, have been linked to the formation of ethyl carbamate, a known carcinogen. This has caused some concern about addition of supplements to juice, and the presence of excess nitrogen in finished wine. Ethyl carbamate arises from several sources in fermented foods, including the reaction of ethanol with urea (Henschke and Jiranek, 1993) or naturally occurring carbamoyl phosphate (Ough, 1976a). Although urea is no longer used in the wine industry as a nitrogen source, it is a byproduct of arginine catabolism in several strains of *Saccharomyces cerevisiae*. Under these conditions, arginine is degraded by the enzyme arginase into ornithine and urea. Other wine processing chemicals have

been implicated as well. Fears that diethyl dicarbonate, DEDC, a cold sterilant, would cause ethyl carbamate formation (Ough, 1976b) resulted in its restriction.

Because supplements are a rich source of ethyl carbamate precursors, it would seem that nitrogen supplementation might lead directly to ethyl carbamate formation. However, when Ingledew and co-workers (1987a) investigated several yeast foods, including DAP, yeast extract, casamino acids and urea, they found that the addition of nitrogen sources to juice did not promote ethyl carbamate formation. Urea itself led to ethyl carbamate formation only in the presence of heat, which may explain its presence in foods fermented at higher temperatures, such as sauerkraut. Henschke and Ough (1991) found that urea did not accumulate in wine because it is utilized as a nitrogen source when fermentation is preceded by an initial aerobic period. In addition, they found that supplementing with excess ammonia (1000 mg/L) promoted urea utilization during fermentation.

2.2.5 *Yeast Strain*

The selection of an appropriate yeast strain is critical in ensuring a complete fermentation (Ough and Groat, 1978). Often strains differ in the time that they take to ferment, their ability to flocculate, the production of sulfur compounds, and the production of aromatic esters (Soles and Ough, 1982). Basic differences between strains are exaggerated in conditions of low nitrogen (Jiranek *et al.*, 1991) when compared to behavior in adequate

nitrogen musts. In addition, the fermentation bouquet that is characteristic of any given yeast strain is affected by the nitrogen source available to the yeast (Rapp and Versini, 1991); the metabolites of some yeasts may negatively affect the aroma profile under conditions of nitrogen starvation.

There may be several reasons for increased fermentation efficiency between strains in different nitrogen conditions. Different yeast strains have different capacities to take up nitrogen. This ability is affected by the presence of other (perhaps spontaneous) wild yeast fermentations occurring in the juice at the same time (Goni and Azpilicueta, 1999).

2.3 ORGANOLEPTIC PROPERTIES OF FINISHED WINE

Finished wine aromas arise from many different sources: from the grape itself, from processing (skin contact, pressing), from fermentation (primary yeast fermentation and malolactic bacterial fermentation), and from aging and maturation (oak contact and the presence of yeast sediment). Fermentation aromas include yeast metabolites: higher alcohols, acids, esters, aldehydes, ketones, and sulfur compounds (Rapp and Versini, 1991). The development of these compounds is influenced both by the presence of aroma precursors (some of which are amino acids) and by the nutritional status of the wine.

No clear relationship is yet established between particular nitrogenous compounds and wine quality. Amelioration of musts with diammonium phosphate has been found to have a positive correlation with the

development of ethyl esters (Herraiz and Ough, 1993) to decrease the development of higher alcohols (2-methyl-propanol-1, 3-mehtyl-butanol-1 and hexanol) (Rapp and Versini, 1991). These changes are thought to improve the overall organoleptic properties and quality of the finished wine. However, Juhász and Törley (1985) found that increased amino acid and arginine content coincided with a decreased hedonic rating. In addition, a positive correlation has been observed between the amino acids tyrosine and phenylalanine and the enhancement of undesirable metabolites of malolactic bacteria, including isobutyric acid and isovalerianic acid (Guitart *et al.*, 1999). Hernández-Orte *et al.*, (1999) found that the best Tempranillo wine was made during years with the lowest total nitrogen, but when the proportion of free α-amino acid nitrogen was the highest.

2.4 FRUIT

2.4.1 VINEYARD MANAGEMENT PRACTICES

Many of the factors that affect the level of nitrogen in the fruit can be controlled in the vineyard. Huang and Ough (1989) found that rootstocks had an effect on the amino acid content of two different grape varieties. Bell and others (1979) found that application of a nitrogenous fertilizer to vineyard rows during the winter resulted in increased petiole nitrogen

both at bloom and veraison compared to the control, in which petiole nitrate had decreased at veraison.

Strategic application of nitrogen in the vineyard may help increase the fruit nitrogen available at harvest (Conradie and Saayman, 1989), but the results vary with timing (Dukes *et al.*, 1991), soil condition, and vine nitrogen status (Spayd *et al.*, 1991). In addition, nitrogen fertilization has been shown to increase soluble solids and lower titratable acidity (Morris *et al.*, 1983), and increase terpene and ester content (Webster *et al.*, 1993). However, nitrogen fertilization can lead to high vine vigor and canopy density (Bell and Robson, 1999). This can lower fruit quality (Smart, 1991) by increasing yield (Ahmedullah and Roberts, 1991) and the risk of bunch rot caused by *Botrytis cinerea*, and decreasing the sugar and anthocyanin content.

2.4.2 PROCESSING

Many of the processing treatments to which grapes are subjected have an effect on the fermentation rate. Ayestarán and co-workers (1995) observed a difference in the fermentation rate of juices clarified by two methods, vacuum filtration and static sedimentation. They observed that the concentration of ammonia nitrogen stayed the same but amino nitrogen increased as protein nitrogen decreased. This suggests proteolytic activity stimulated by the centrifugation treatment. Ferrando *et al.* (1998) showed that three different clarification treatments (vacuum filtration,

centrifugation and settling) did not affect the ammonium nitrogen or total free amino acid content of juice. However, vacuum filtration lowered solids content and significantly lengthened fermentation time, possibly because of the removal of other yeast nutritional components.

2.4.3 *Nitrogen Status of Fruit at Harvest*

Amino acids account for 60-90% of the nitrogen found in grape juice (Kliewer, 1969). 59-90% of this nitrogen comes from eight amino acids: α-alanine, γ-amino-butyric acid, arginine, aspartic acid, glutamic acid, proline, serine and threonine; in most varieties of *Vitis vinifera*, including Chardonnay, proline is the predominant amino acid (Kliewer, 1970). During the final stages of maturation, proline accumulation increases dramatically as ammonia, arginine and other amino acids begin to level out or drop (Kliewer, 1968). However, Hernández-Orte and others (1999) found that arginine increased during ripening, and was well correlated with the accumulation of soluble solids.

A continuing survey of Oregon grape must and juice conducted by the Oregon State University Enology Extension Laboratory has assessed the amino acid and ammonia profiles of Willamette Valley Pinot Noir, Chardonnay, Pinot Blanc, and Pinot Gris during the 1996-1999 vintages (Watson, 2000a) ***(Table 2.1)***. In 1998, the average yeast assimilable nitrogen content of wines submitted for analysis from a group of Willamette Valley winery cooperators was 159 mg N/L (59 samples), and in 1997 the average

was 178 mg N/L (58 samples). While these averages are above the minimum recommended nitrogen content of 140 mg N/L, certain varietals, such as Chardonnay, were more likely to be nitrogen deficient. In 1997, 80% of Chardonnay was less than 140 ppm. In 1999, when fruit was harvested late and achieved a high level of maturity, only two of the submitted industry samples were deficient in nitrogen. HPLC of Chardonnay juice from 1999 ***(Figure 2.1)*** show the distribution of YANC compounds at harvest (Note: proline was not included in this analysis).

When amino acid and ammonia levels in Oregon Pinot Noir and Chardonnay were monitored throughout ripening (Watson *et al.*, 2000b) during the 1999 vintage, it was possible to see a steep increase in amino acid levels during the final stages of maturation. During the final two weeks before harvest, amino acids increased dramatically as ammonia levels decreased. This observation contrasts with the findings of Kliewer (1968) who observed amino acids leveling off during the late stage of maturation. These discrepancies likely reflect climate and maturity differences in Oregon and California.

2.4.4 METHODS OF ANALYSIS

Analysis of nitrogen compounds has historically been cumbersome and expensive. For wineries to use YANC analysis as a tool for predicting sluggish fermentation and calculation of supplement additions, a simple test is needed that can be used on the premises with minimal equipment.

Table 2.1 *YANC content of grapes provided by a group of Oregon winery cooperators. Data from Watson* et al., *1998-2000.*

	1997				1998				1999			
Grape	number of samples	range, mg N/L	mg N/L average	less than 140 mg N/L	number of samples	range, mg N/L	mg N/L average	less than 140 mg N/L	number of samples	range, mg N/L	mg N/L average	less than 140 mg N/L
Chardonnay	15	45-211	109	80%	14	65-166	108	79%	6	50-247	131	17%
Pinot Gris	35	77-442	210	40%	41	77-482	177	34%	4	141-281	143	0%
Pinot Blanc	5	69-267	136	60%	2	81-151	116	50%	2	165-236	201	0%
Pinot Noir	3	174-274	209	0%	2	151-212	182	0%	35	115-346	204	6%
Total	58	45-442	178	50%	59	65-482	159	30%	47	50-346	170	6%

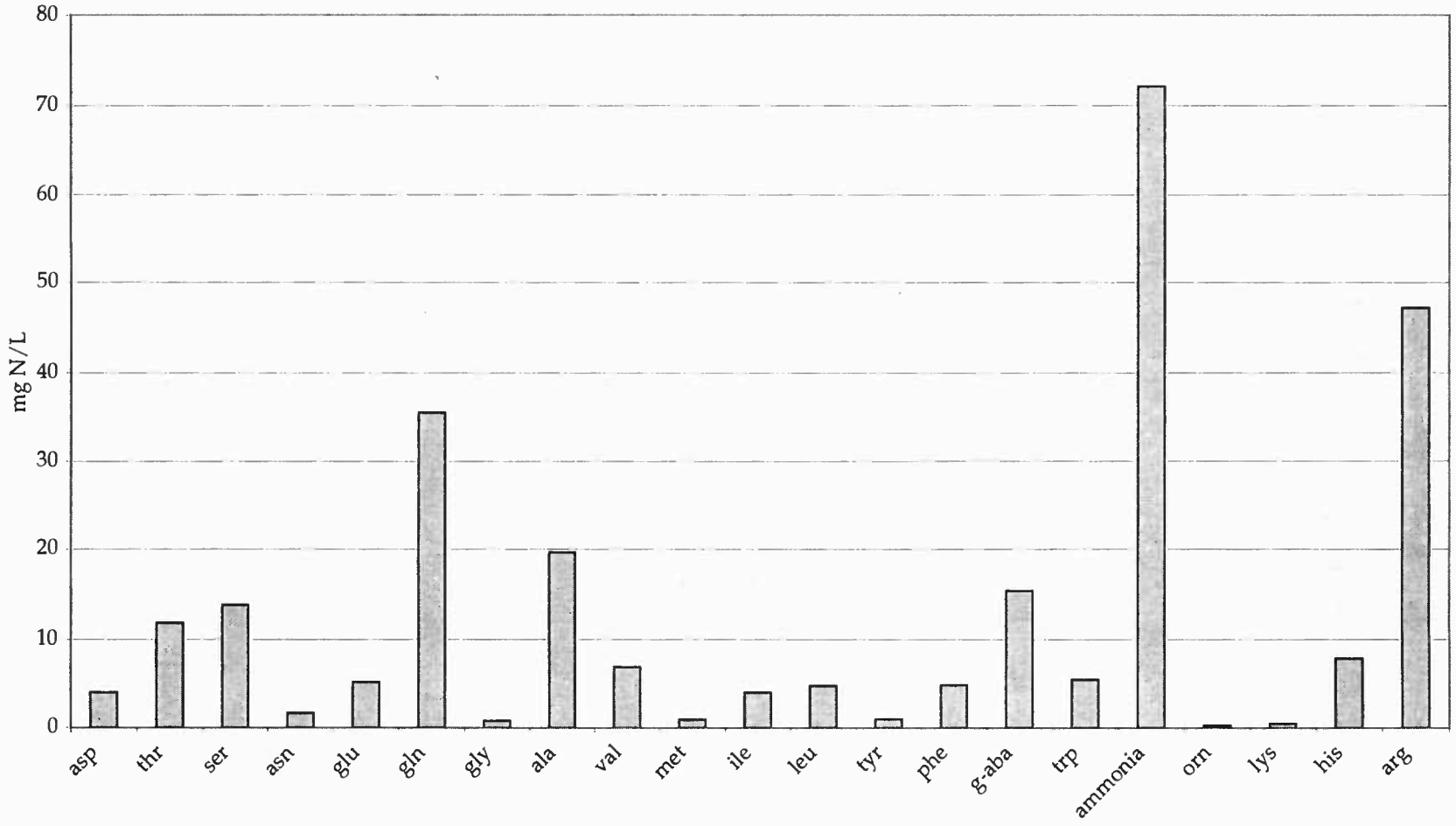

Figure 2.1 *Average N concentration (HPLC) in three Oregon Chardonnay juices, 1999. Proline was not measured. Unpublished data, Watson* et al., *2000.*

Nitrogen profiles change rapidly in maturing fruit and it is necessary to correct nitrogen deficits early, yet avoid over-supplementing juices that contain sufficient fermentable nitrogen.

It is important for an assay to estimate only assimilable nitrogen. Usually, assays that measure assimilable nitrogen employ a method for the derivatization of alpha-amino acids. The *o*-phthaldialdehyde (OPA) derivatization, which detects primary amines, has been used extensively (Wong *et al.*, 1985; Ishida *et al.*, 1981; Jones *et al.*, 1981; Sanders and Ough, 1985; Dukes and Butzke, 1998). Crowell and others (1985) used the TNBS method, in which 2, 4, 6-trinitrobenzene sulfonic acid reacts with primary amines. This method, although reasonably accurate when used in musts, requires the removal of large peptides and proteins, a time-consuming preparation. The ninhydrin procedure for determining free amino nitrogen (FAN) has been used in worts and beer (Jones and Rainbow, 1966); however, this method is tedious and inaccurate (Montiero and Bisson, 1991), as it tends to overestimate YANC because it measures proline.

HPLC (high-pressure liquid chromatography) is an accurate and fast method for identifying fluorescent amino acid derivatives. Sanders and Ough (1985) used HPLC to separate and quantify OPA-derivatized amino acids in white wine in less than 40 minutes. HPLC is advantageous because of its high recovery and short run time; in addition, it can be used to identify small OPA-derivatized peptides (Bartolomé *et al.*, 1997). However,

HPLC equipment is expensive and unsuited for routine use in most wineries.

Montiero and Bisson (1991) estimated nitrogen content of grape must with a bioassay based on final cell density of a yeast culture, measured by absorbance at 580 nm. This method had good correlation with amino acid analysis, especially those nitrogen sources thought to be important for yeasts: ammonium ion, glutamine, glutamate, asparagine, aspartate, arginine, serine and alanine. However, this assay takes several days, and is more suited to diagnosis of problem fermentations, rather than prevention.

Fortunately, recent research has established a simple, fast, spectrophotometric assay to detect primary amines. Dukes and Butzke (1998) use *o*-phthaldialdehyde derivatives, which have UV absorbance proportional to primary amine concentration. This assay has been termed "NOPA" (for nitrogen by OPA) and is used to determine primary amines in juice and wine. This assay is relatively inexpensive, and currently available in kit form. NOPA detects peptides and proteins, however, the error due to protein and peptide concentration is relatively small (Dukes and Butzke, 1998). NOPA does not measure proline.

Ammonia has been measured using electrical conductivity (Carlson, 1978) in samples prepared by the Kjeldahl procedure. A much simpler method of analysis is available from Sigma, which makes a plasma ammonia kit that is appropriate for detecting ammonium ion in wine and

diluted grape juice samples. This procedure is based on the reduction of NADH by L-glutamate dehydrogenase, a reaction that is stoichiometric to the amount of ammonia in the sample.

2.5 SUPPLEMENTS

2.5.1 DIAMMONIUM PHOSPHATE (DAP)

Ammonium ion is the most easily assimilated of all the nitrogen sources, and is depleted quickly by yeast during growth phase. Yeasts are able to use ammonium sulfate, ammonium chloride, and diammonium phosphate (Montiero and Bisson, 1991). DAP is inexpensive and is commonly used throughout the wine industry, as it is also a good source of phosphate.

Montiero and Bisson (1992a) found that supplementation with DAP had a significant effect on biomass accumulation for Prise de Mousse but not in UCD 522, although it increased rates of fermentation in both commercial yeast strains. High levels (200 g/hL) of DAP decreased the level of arginine consumption, presumably due to the repression of amino acid transporters by ammonia (see 2.2.2.4). They found that higher levels of DAP supplementation (treatments in which less arginine was utilized) produced wines with high levels of amino acids present at the end of fermentation.

2.5.2 *Yeast Extracts and Preparations*

When yeast cells are autolyzed, the two fractions, yeast extracts and yeast hulls, or ghosts, are both used for supplementing wine. Yeast extracts contain intracellular components, especially proteins and nucleic acids, which may add nitrogen compounds to juice. O'Connor-Cox and co-workers (1991) found that addition of yeast extract to wort increased growth rate and cell mass, leading to increased ethanol yields. Ingledew and others (1986) conducted a survey of the components of several yeast foods, including yeast extracts, hydrolyzed proteins, and other chemicals, which indicated that the manufacturer's product sheets were often uninformative or misleading in their compositional claims.

2.5.3 *Yeast Hulls*

While not a source of nitrogen, yeast hulls are often added to improve fermentation. They add survival factors (see 2.2.2.5) that increase the resistance of the cell membrane to alcohol leakage. This was shown by Munoz and Ingledew (1989a), who compared the rate of fermentation with yeast hulls with that of a fermentation containing the lipid extracted from the yeast hulls. It had been previously proposed that yeast hulls might act by adsorbing toxic fatty acids (decanoic and octanoic), which are a byproduct of yeast metabolism (Lafon-Lafourcade *et al.*, 1984). However, Munoz and Ingledew (1989b) found no apparent adsorbing effect from yeast hulls. They

felt that the beneficial effect came from the addition of lipids, such as Tween 80 and ergosterol. They therefore concluded that the stimulatory effect seen with yeast hulls was due to the addition of unsaturated fatty acids (C16 and C18), which acted as oxygen substitutes, thereby preventing oxygen deficiency-related sticking by increasing cell number.

3. MATERIALS AND METHODS

3.1 SAMPLE PRODUCTION

Chardonnay juice from the 1997 vintage was obtained from the Adams Block at Rex Hill Vineyards in Dundee, Oregon. The fruit, heavily infected with rot and mold at harvest, had been pressed, treated with pectolytic enzymes, settled overnight and DE filtered. Approximately 100 gallons of juice was transported to Oregon State University where it was treated with 25 ppm SO_2 and frozen (-35° F) until used.

The juice was thawed in October 1998. SO_2 adjustments were made (35 ppm total SO_2) and the juice divided into 28 three-gallon lots. Additions (see Table 3.1) for two replications of 14 treatments were weighed and carefully dissolved in a small amount of juice before addition. Samples were taken before and after supplement addition and frozen for later analysis. The juice was inoculated with Lalvin CY 3079 yeast at a level of 1 gram per gallon (25 g/hL). Fermentation was conducted at a controlled temperature (19° C). Hydrometer readings were taken every 48 hours to determine fermentation rate. Samples, corresponding to the hydrometer readings, were taken at two-day intervals and frozen for later analysis.

Fermentation was considered finished when the Brix readings reached -1.8° Brix. The wines were racked and 20 ppm SO_2 added within 1-2 days of the end of fermentation. Because the treatments finished fermenting at different times, total exposure time to the yeast lees was not the same for

every treatment. When the last treatment had been racked, the temperature was lowered to approximately 8° C for cold stabilization. Malolactic fermentation was not promoted. The wines were fined with bentonite and the SO_2 adjusted to 50 ppm total in March 1999. Wines were subjected to a preliminary sensory screening in April, 1999, to determine the plausibility of conducting a descriptive panel. Because of perceived replication differences in sulfur aromas, a full sensory panel was not performed.

Table 3.1 *Treatments applied to 3-gallon lots of Chardonnay juice.*

Treatment number	Additions to 3 gallons	Equivalent additions per 1000 gallons or (g/hL)
1	Control	Control
2	Superfood 5.5 grams	Superfood 4 pounds (50)
3	Superfood 11 grams	Superfood 8 pounds (100)
4	Fermaid K 2.75 grams	Fermaid K 2 pounds (25)
5	Fermaid K 2.75 grams Yeast Hulls 2.75 grams	Fermaid 2 pounds (25) Yeast Hulls 2 pounds (25)
6	Fermaid K 2.75 grams DAP 2.75 grams	Fermaid K 2 pounds (25) DAP 2 pounds (25)
7	Fermaid K 2.75 grams DAP 5.5 grams	Fermaid K 2 pounds (25) DAP 4 pounds (50)
8	DAP 2.75 grams	DAP 2 pounds (25)
9	DAP 5.5 grams	DAP 4 pounds (50)
10	DAP 11 grams	DAP 8 pounds (100)
11	DAP 5.5 grams Yeast hulls 2.75 grams	DAP 4 pounds (50) Yeast Hulls 2 pounds (25)
12	DAP 5.5 grams PL-50 1.8 milliliters	DAP 4 pounds (50) PL-50 1 pound (12.5)
13	DAP 5.5 grams Thiamine 6 milligrams	DAP 4 pounds (50) Thiamine 2 grams (0.6 mg/L)
14	DAP 5.5 grams Thiamine 6 milligrams Yeast Hulls 2.75 grams	DAP 4 pounds (50) Thiamine 2 grams (0.6 mg/L) Yeast Hulls 2 pounds (25)

Bottling occurred in July, 1999, after adjustment to 35 ppm free SO_2. One bottle from each duplicate replication was sent to Sara Spayd at Washington State University for GC-MS sulfides analysis. Selected frozen samples were sent to Gordon Burns at Enological Testing Services for amino acid analysis. These treatments were two replications each of: Treatment 1 (control) before inoculation, Treatment 1 (control) mid-fermentation, Treatment 1 (control) end of fermentation, Treatment 3 (Superfood 8 lbs/1000 gal, 100 g/hL) before inoculation, Treatment 4 (Fermaid K 2 lbs/1000 gal, 25 g/hL) before inoculation, Treatment 8 (DAP 2 lbs/1000 gal, 25 g/hL) end of fermentation, Treatment 9 (DAP 4 lbs/1000 gal, 50 g/hL) end of fermentation, and Treatment 10 (DAP 8 lbs/1000 gal, 100 g/hL) end of fermentation. This limited set of samples was selected because of the expense of amino acid analysis. A full set of bottled wine samples was also sent to ETS for ethyl carbamate analysis.

3.2 *Nitrogen Analysis*

The yeast assimilable nitrogen content (YANC) of the frozen samples was calculated as the sum of the α-amino acid nitrogen and the ammonia nitrogen. These values were determined using two different spectrophotometric assays: the Sigma Diagnostic Ammonia assay (Sigma 171-B) and the NOPA (nitrogen by ortho-phthaldialdehyde) assay (Dukes and Butzke, 1998) for α-amino acids. These two methods used in

combination give a reasonable estimate of the total yeast assimilable nitrogen content of the must.

Frozen samples from the beginning, middle and end of fermentation from selected treatments were sent for HPLC analysis of amino acids and GC-MS analysis of ethyl carbamate at ETS Laboratories in St. Helena, CA.

GC-MS sulfide analysis of the finished wines was performed at Washington State University, IAREC, Prosser, WA.

3.2.1 AMMONIA ASSAY

Procedure:

Sigma Diagnostics Kit for Ammonia: catalog no. 171-B. Juice samples were diluted volumetrically 1 to 10 with distilled water prior to analysis. Fermenting wine samples were not diluted. Dilution was considered necessary when the concentration of ammonia in the sample was greater than 15 mg ammonia/L, corresponding to a maximum change in absorbance of ~0.5. The spectrophotometer was zeroed with water. The Ammonia Assay Solution was prepared by adding 10 mL distilled water. Ammonia Assay Solution (1 mL) was added to a series of cuvettes containing the duplicate samples (0.1 mL sample), a blank (0.1 mL water) and a 5 mg/L ammonia control (0.1 mL control solution). The cuvettes were covered with parafilm, inverted and allowed to equilibrate for approximately 3 minutes at room temperature. The initial absorbance of

each cuvette (A_i) at 340 nm was recorded. Enzyme was then added (0.01 mL L-Glutamate Dehydrogenase solution) and gently mixed. The final absorbance (A_f) of each cuvette was recorded after 5 minutes.

Calculations:

$[(A_i \text{ sample} - A_f \text{ sample}) - (A_i \text{ blank} - A_f \text{ blank})] \times \text{dilution} \times 30.3 = \text{mg/L ammonia}$

The final concentration of ammonia (mg/L) was converted to the concentration of nitrogen (mg/L) from ammonia by multiplying by 0.8235.

3.2.2 NOPA ASSAY

The procedure for the NOPA (nitrogen by o-phthaldialdehyde) assay is a modified version of that used by Butzke and Dukes (1998).

The reagent solution consisted of 0.671 g OPA dissolved and made to 100 mL with 95% vol ethanol. This OPA solution was added to a 1000-mL volumetric flask containing an aqueous solution of 3.837 g NaOH (s), 8.468 g of ortho-boric acid (s) and 0.816 g NAC (s). The solution was made to volume with deionized water.

A standard curve (0-140 mg N/L) was made using 10 mM isoleucine stock (0.328 g ile (s) in 250 mL deionized water). The linear regression equation of this curve was used to determine the concentrations of nitrogen from the net absorbance of the sample.

The spectrophotometer was zeroed with a blank consisting of 50 µL of water and 3 mL OPA reagent in a standard 4.5-mL UV-grade cuvette.

The juice was prepared by cold settling. A dilution of the juice was used if the nitrogen concentration grossly exceeded the range of the standard curve (>200 N mg/L). 50 µL of the juice sample was pipetted into a cuvette. The sample was mixed with 3 mL of the OPA solution, and the absorbance at 335 nm was read after 10 minutes.

4. Results and Discussion

4.1 Finished Wine Components

Table 4.1 shows basic analysis of the finished, supplement-treated wines. All treatments had "dry" levels of residual sugar, averaging less than 0.4%, indicating that none of the fermentations stuck. Volatile acidity, alcohol, pH, titratable acidity, malic acid, and sulfur dioxide are all within normal ranges.

4.2 NOPA and Ammonia

The unsupplemented control juice contained 46 mg N/L ***(Table 4.2)***. The diammonium phosphate treatments increased the fermentable nitrogen content by about 20-25 mg N/L per 1 lb/1000 gallons (12.5 g/hL) added. Superfood and Fermaid K increased the ammonia nitrogen fraction by approximately 8-10 mg N/L per 1 lb/1000 gallons (12.5 g/hL) addition. Superfood at 4 and 8 lbs/1000 gallons (50 and 100 g/hL) increased the alpha amino acid content slightly, while Fermaid K at 2 lbs/1000 gallons (25 g/hL) provided no detectable increase in alpha amino acid content. PL-50 yeast extract at 1 lb/1000 gallons (12.5 g/hL) increased the assimilable nitrogen content from alpha amino acids from 40 to 58 mg N/L.

4.3 FERMENTATION RATE

There was an inverse relationship between the days to dryness and the amount of fermentable nitrogen present in the juice prior to fermentation ***(Figure 4.1)***. The rate of fermentation, estimated by the fermentation curves between days 7 and 15 of fermentation ***(Figure 4.2)*** increased with higher levels of fermentable nitrogen, reaching a maximum rate at approximately 150 mg N/L YANC. DAP at 4 lbs/1000 gallons (50 g/hL) fermented more quickly than Superfood at 4 and 8 lbs/1000 gallons (50 and 100 g/hL) and Fermaid K at 2 lbs/1000 gallons (25 g/hL) (with or without the addition of 2 lbs/1000 gallons (25 g/hL) of DAP or 2 lbs/1000 gallons (25 g/hL) of yeast hulls) ***(Figure 4.2)***. DAP at 4 lbs/1000 gallons (50 g/hL) (with the addition of yeast hulls, thiamine, Fermaid K or PL-50) was nearly as effective as 8 lbs/1000 gallons (100 g/hL) of DAP. The control wine did not stick, but took 42 days to ferment to "dryness".

Table 4.1 *Basic analysis of new wines.*

Sample	*Alcohol %*	*Residual Sugar g/100 ml*	*Volatile Acidity g/100 ml*	*pH*	*Titratable Acidity g/100 ml*	*Malate (g/L)*	*SO_2 free/total ppm*
T1R1	11.9	.26	.032	3.00	0.70	1.6	13/51
T1R2	11.8	.27	.027	3.03	0.68	2.8	13/48
T2R1	11.9	.19	.021	3.01	0.69	3.0	11/48
T2R2	11.9	.22	.019	3.01	0.67	2.3	11/48
T3R1	11.9	.09	.017	3.03	0.66	2.7	13/48
T3R2	12.0	.11	.022	3.01	0.65	2.8	13/51
T4R1	11.9	.19	.024	3.04	0.66	2.6	15/48
T4R2	11.7	.31	.015	3.01	0.66	2.4	13/46
T5R1	11.7	.44	.025	3.01	0.67	2.3	11/48
T5R2	11.7	.36	.027	3.03	0.68	2.2	12/46
T6R1	11.9	.18	.016	3.02	0.68	2.4	13/38
T6R2	12.0	.14	.017	2.99	0.66	2.2	13/44
T7R1	11.7	.30	.014	3.01	0.67	2.1	12/44
T7R2	11.9	.32	.008	3.03	0.65	2.4	13/44
T8R1	11.7	.36	.014	3.01	0.66	2.5	15/51
T8R2	11.7	.36	.016	3.03	0.66	2.7	13/48
T9R1	11.9	.34	.012	3.02	0.65	2.5	12/46
T9R2	11.5	.34	.011	2.98	0.67	2.8	13/51
T10R1	11.9	.29	.007	2.96	0.65	2.5	13/48
T10R2	11.9	.46	.010	3.01	0.65	2.3	13/48
T11R1	11.7	.14	.010	3.04	0.66	2.6	15/45
T11R2	11.9	.10	.014	3.01	0.68	2.7	13/44
T12R1	11.5	.10	.013	3.02	0.67	2.7	11/44
T12R2	11.9	.18	.012	3.02	0.68	2.5	13/48
T13R1	11.6	.14	.014	3.01	0.65	2.6	15/51
T13R2	11.9	.12	.015	3.02	0.69	2.6	13/48
T14R1	11.7	.16	.012	3.01	0.65	2.1	11/48
T14R2	11.7	.14	.010	3.04	0.66	2.4	13/44

Table 4.2 *Yeast assimilable nitrogen content and fermentation times of nutrient supplemented Chardonnay.*

Treatment*		mg N/L NOPA	mg N/L as NH3	mg N/L YANC	Days to dryness
T1	Control	40	6	46	42
T2	Superfood 4 lbs	46	31	77	29
T3	Superfood 8 lbs	49	59	108	25
T4	Fermaid K 2 lbs	38	21	59	33
T5	Fermaid 2 lbs + Yeast Hulls 2 lbs	46	17	63	33
T6	Fermaid K 2 lbs+ DAP 2 lbs	41	65	106	25
T7	Fermaid K 2 lbs+ DAP 4 lbs	42	110	152	19
T8	DAP 2 lbs	43	52	95	25
T9	DAP 4 lbs	43	86	129	23
T10	DAP 8 lbs	46	176	222	17
T11	DAP4 lbs+ 2 lbs Yeast Hulls	42	90	132	19
T12	DAP 4 lbs+ PL-50 1 lb	58	95	153	19
T13	DAP 4 lbs+ 2 g Thiamine	46	100	146	21
T14	DAP 4 lbs + 2 g Thiamine + 2 lbs Yeast Hulls	48	88	136	19

*average of duplicate lots

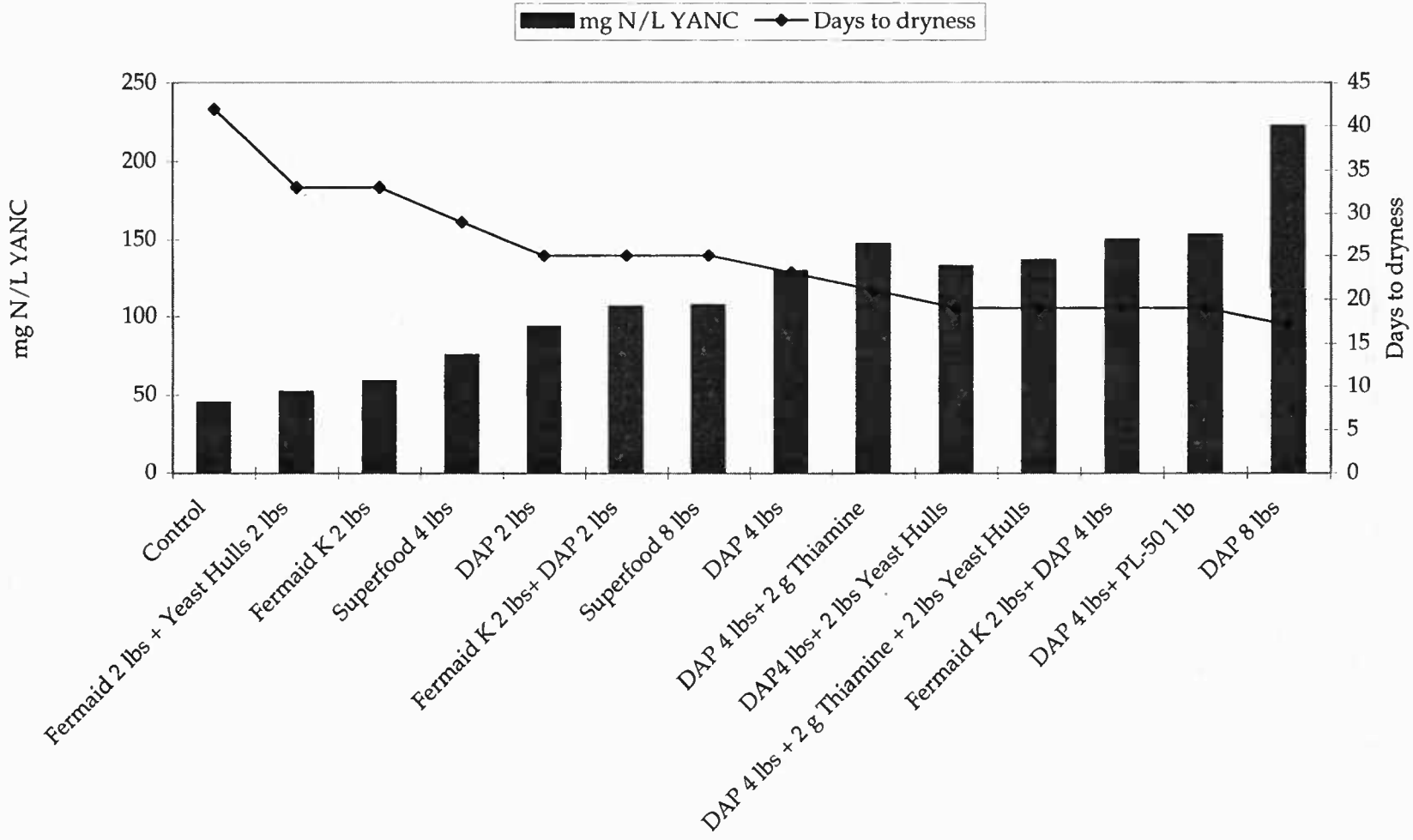

Figure 4.1 *The inverse relationship between the length of fermentation and the YANC level.*

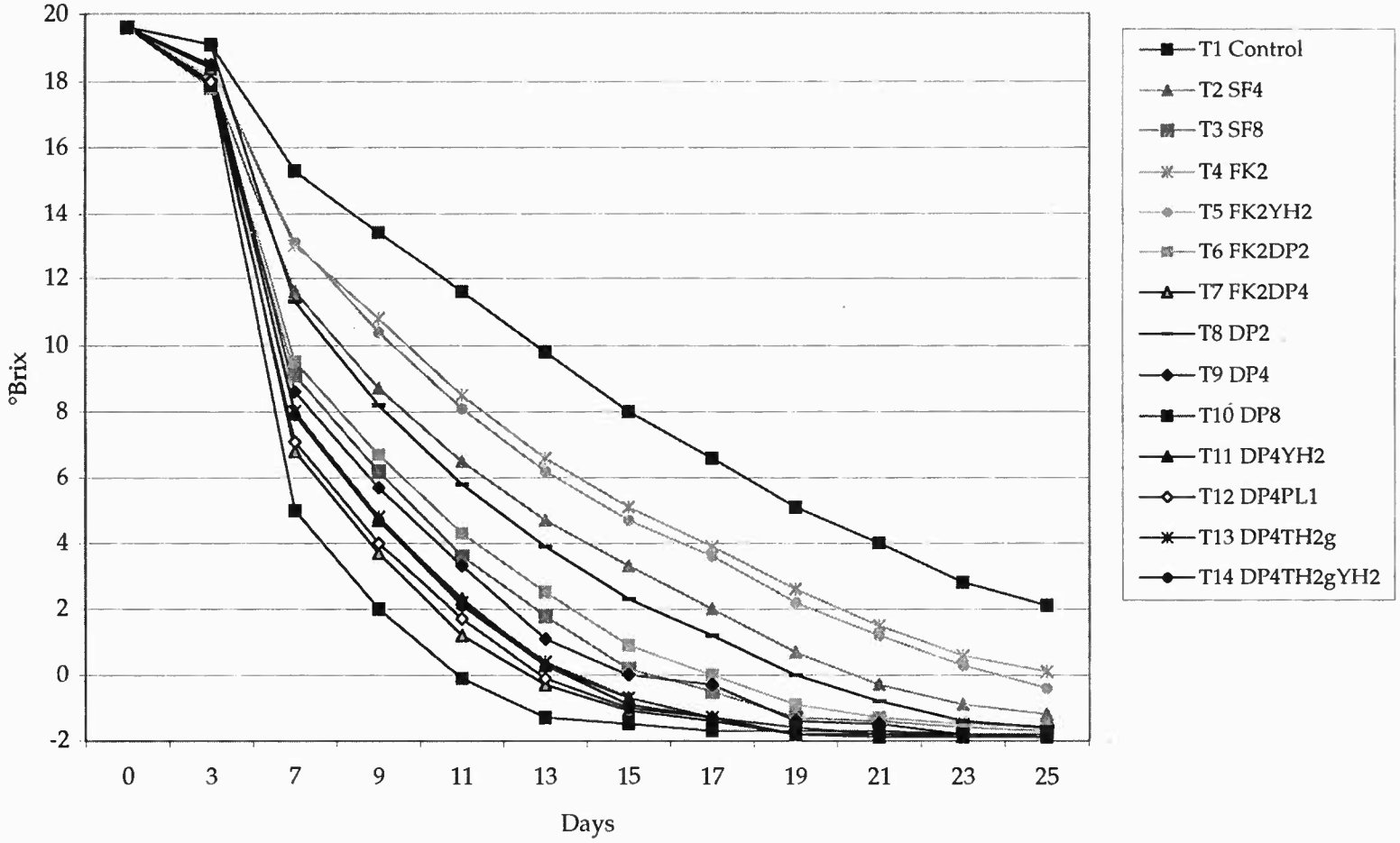

Figure 4.2 *Fermentation curves for supplement-treated Chardonnay. For clarity, only one replication is shown per treatment. Dryness (-1.8°Brix) is not shown for all treatments.*

Ammonia was depleted rapidly upon the onset of fermentation to undetectable levels in all treatments ***(Figures 4.3.1-4.3.14)***. Amino acids decreased to a low of approximately 10 mg N/L early in fermentation, but then increased to between 20 and 50 mg N/L by the end of fermentation, varying with treatments. This is presumably due to leakage or excretion from cells. The addition of the legal limit of DAP (8 lbs/1000 gallons) (100 g/hL) produced wines with the highest alpha amino acid content (as measured by NOPA) at the end of fermentation, at levels as high or higher than that present in the juice prior to fermentation. The amino acid content in new wines at the end of fermentation increased linearly (r^2 = .7696) with the initial concentration of ammonia nitrogen present in the juice prior to fermentation ***(Figure 4.4)***.

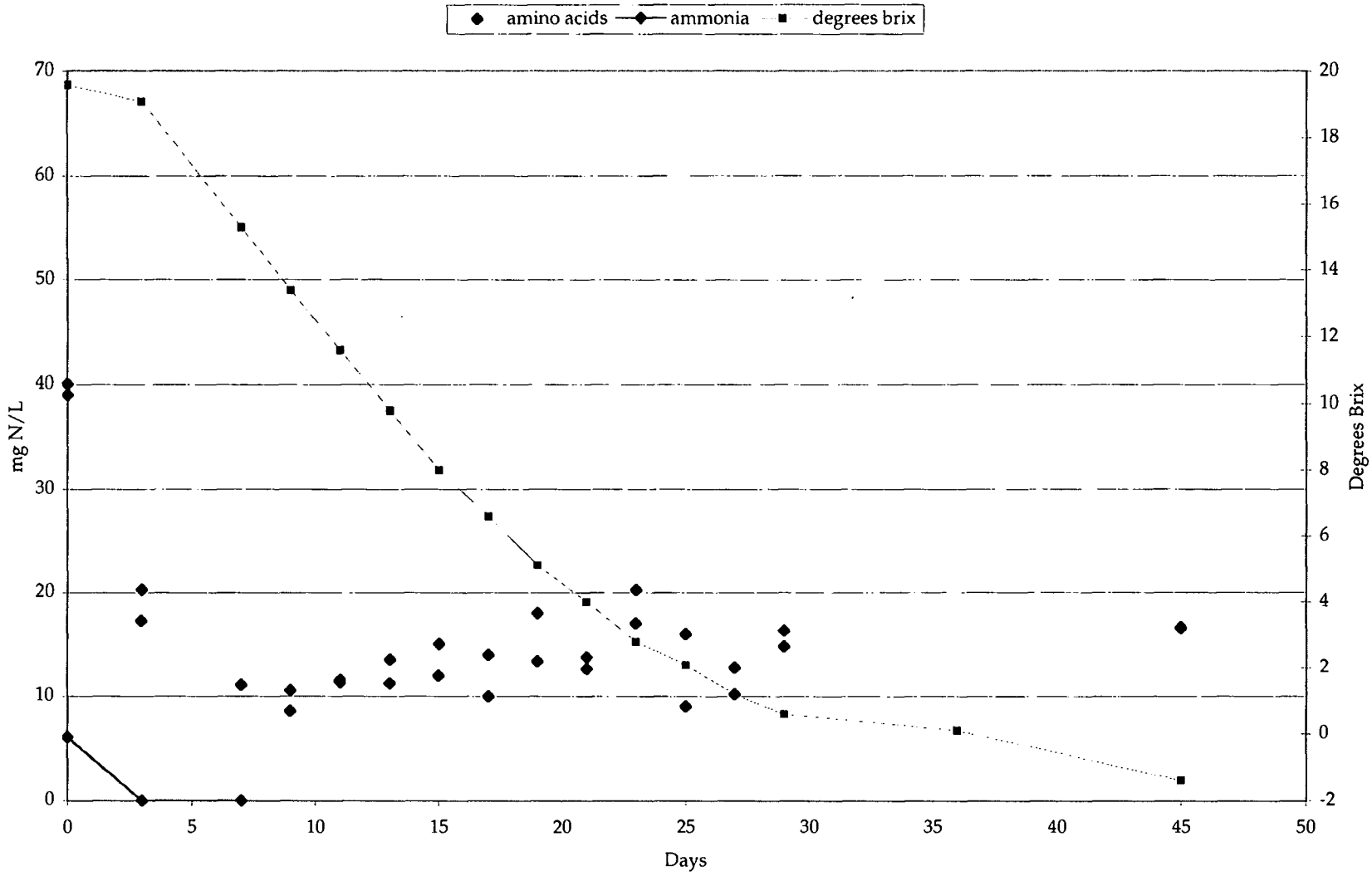

Figure 4.3.1 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 1, Control.

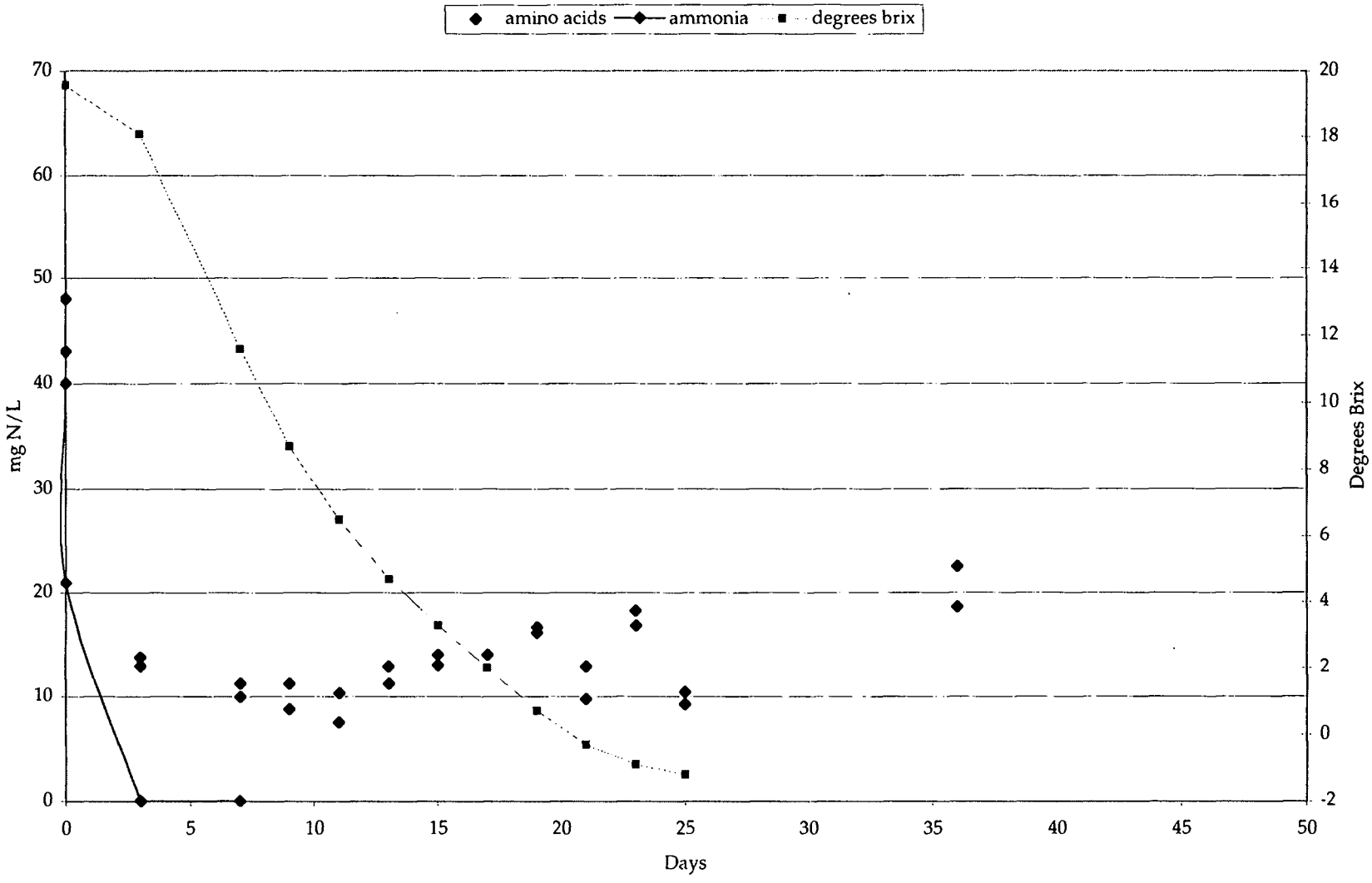

Figure 4.3.2 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 2, Superfood 4 lbs/1000 gallons.

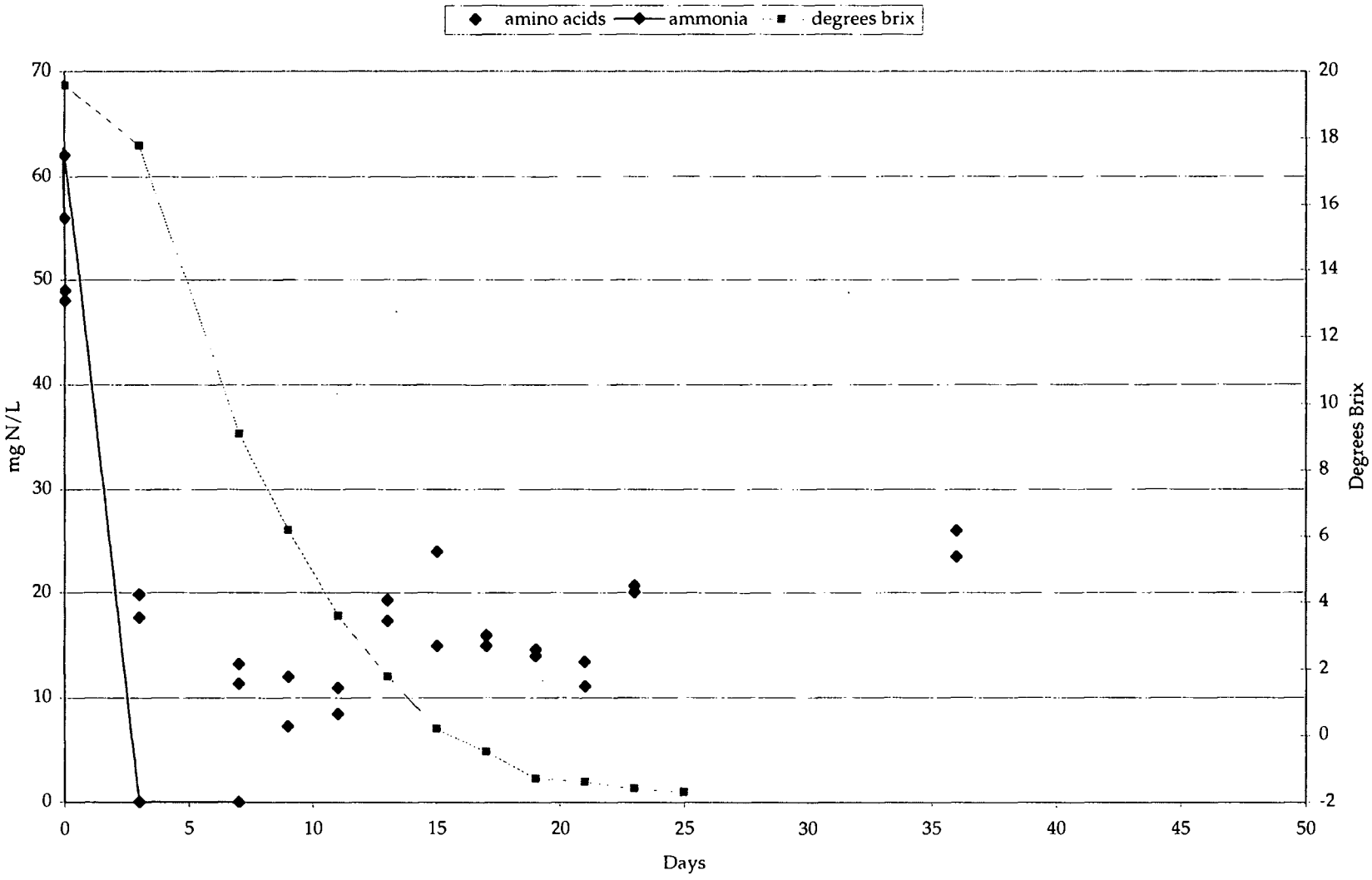

Figure 4.3.3 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 3, Superfood 8 lbs/1000 gallons.

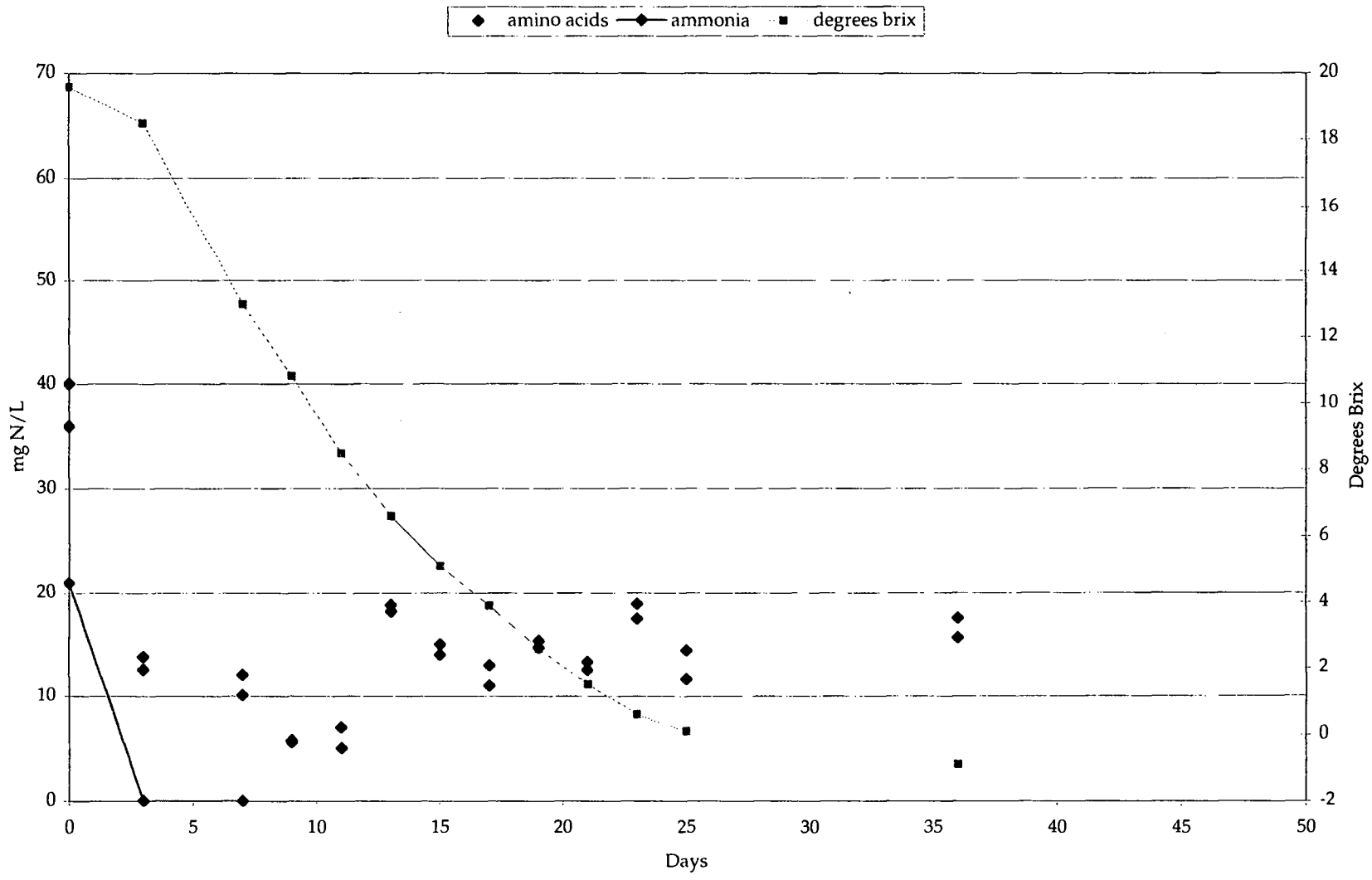

Figure 4.3.4 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 4, Fermaid K 2 lbs/1000 gallons.

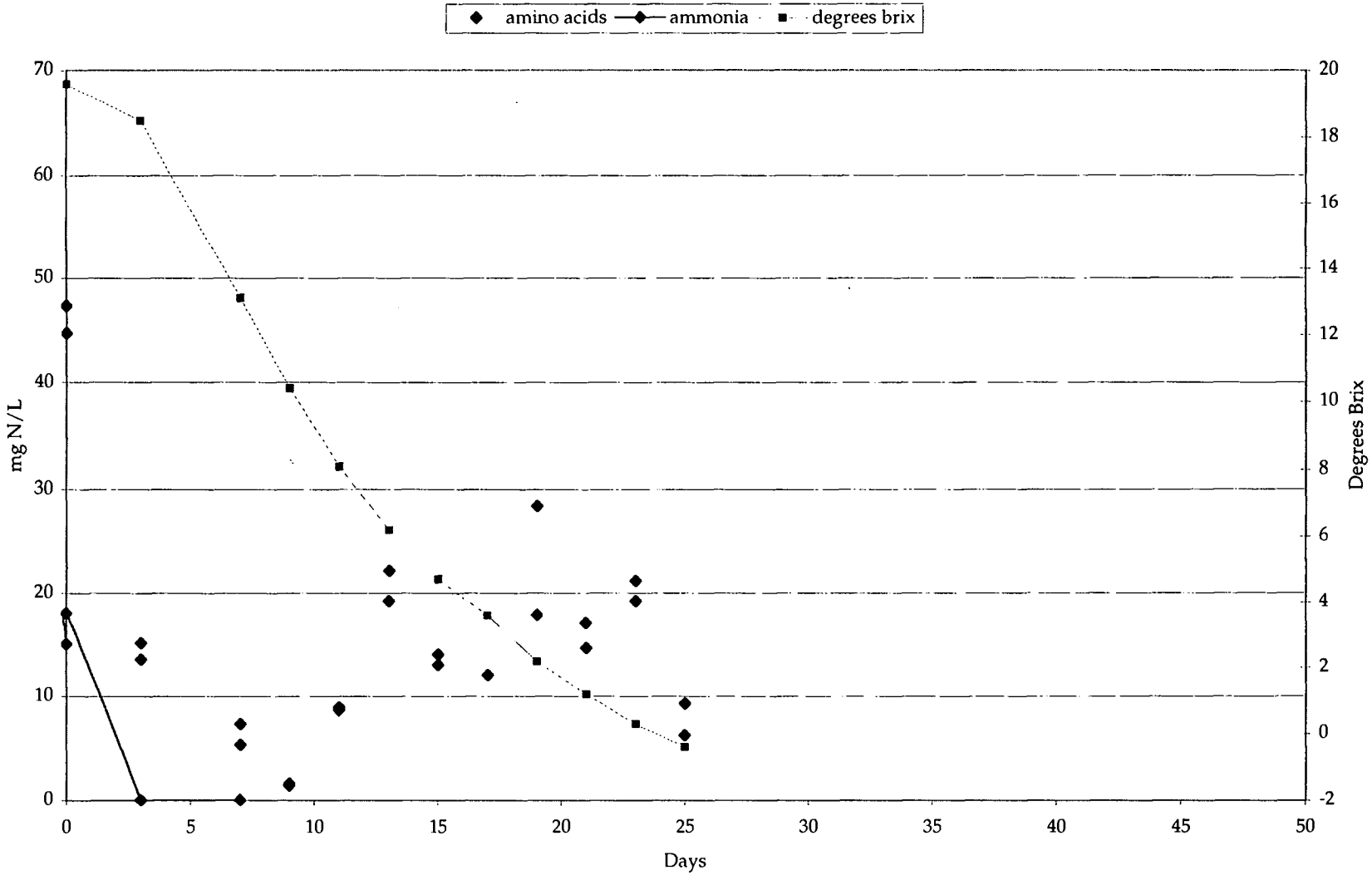

Figure 4.3.5 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 5, Fermaid K 2 lbs/1000 gallons + Yeast Hulls 2 lbs/1000 gallons.

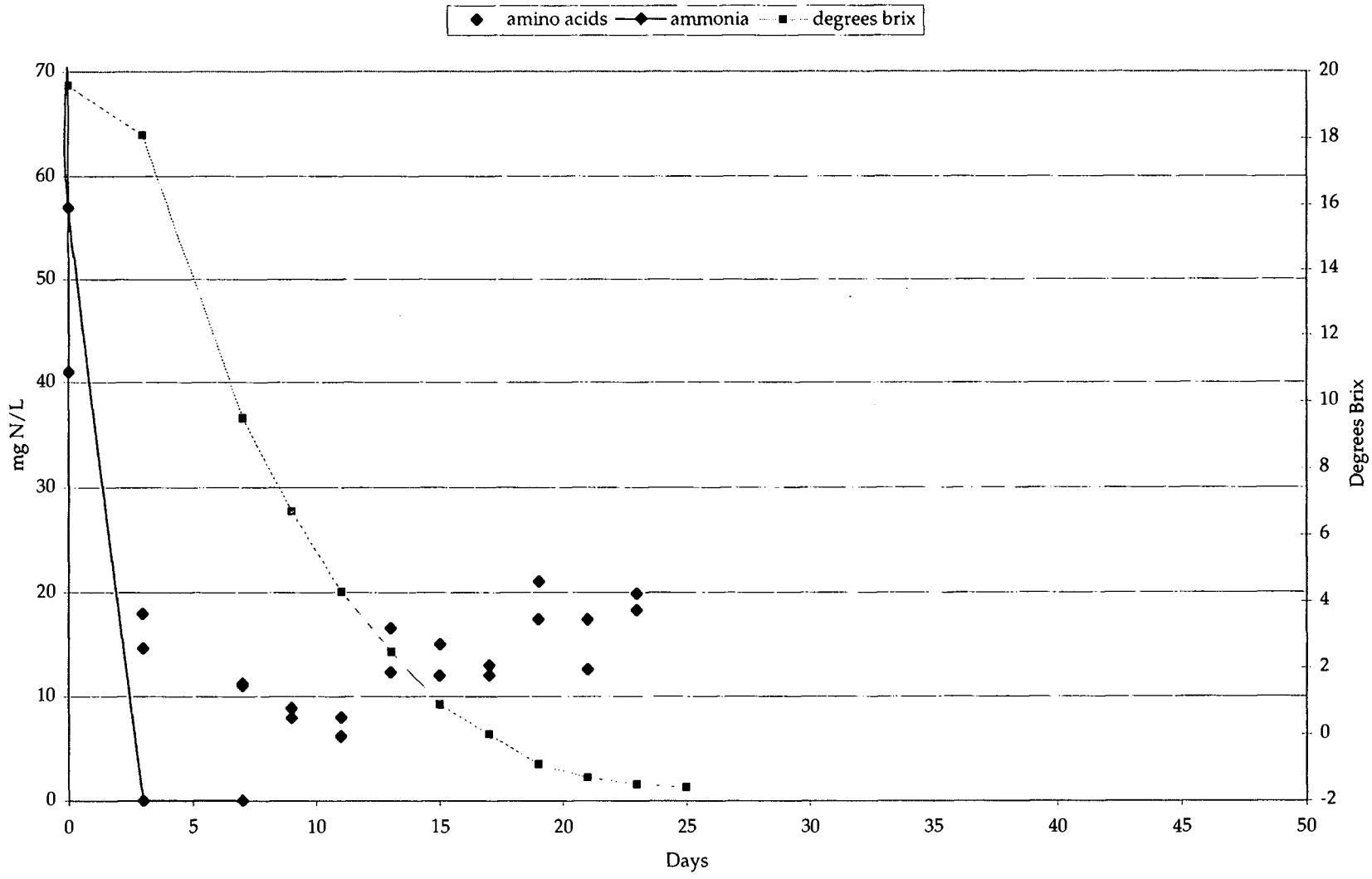

Figure 4.3.6 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 6, Fermaid K 2 lbs/1000 gallons + DAP 2 lbs/1000 gallons.

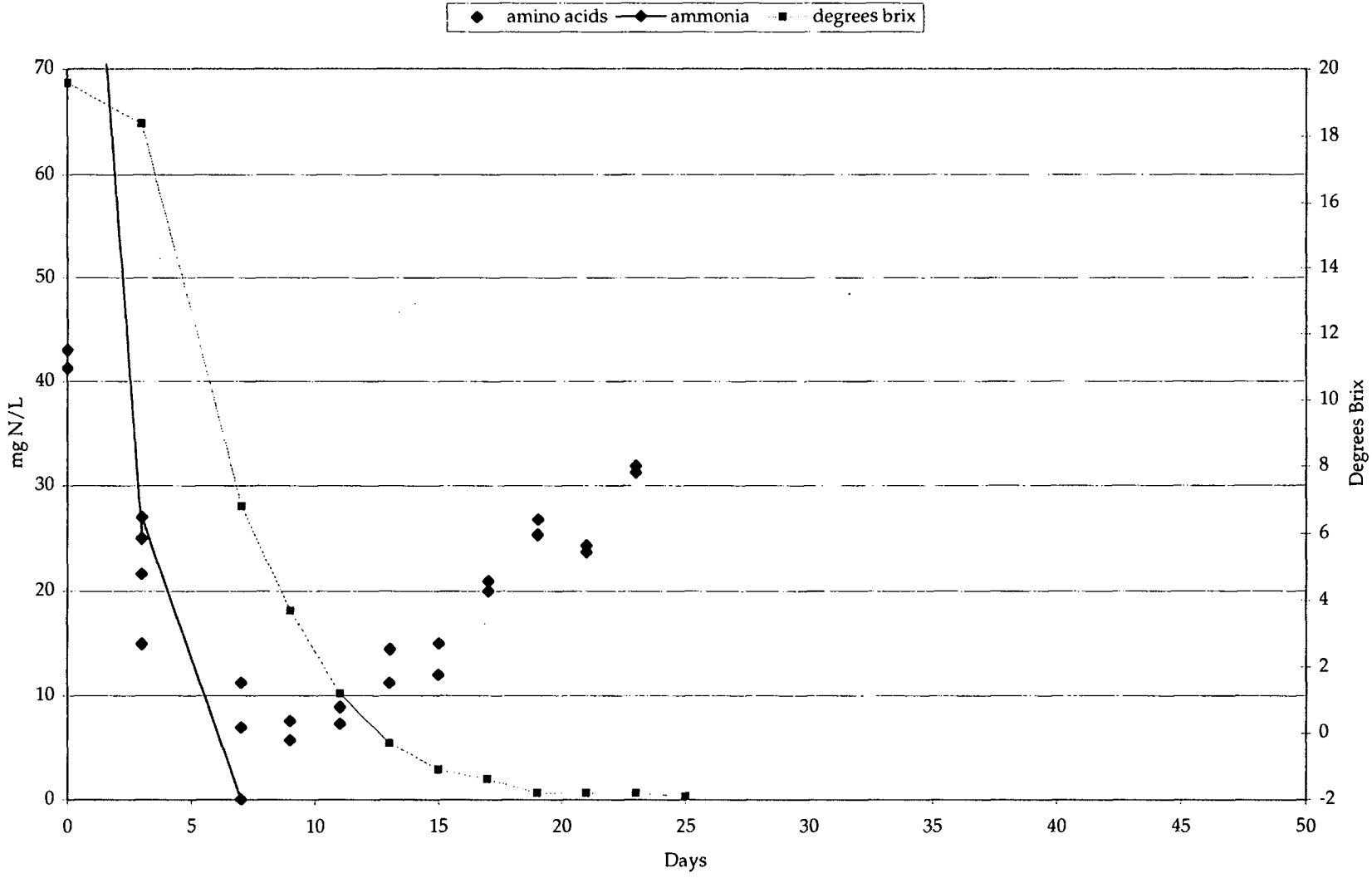

Figure 4.3.7 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 7, Fermaid K 2 lbs/1000 gallons + DAP 4 lbs/1000 gallons.

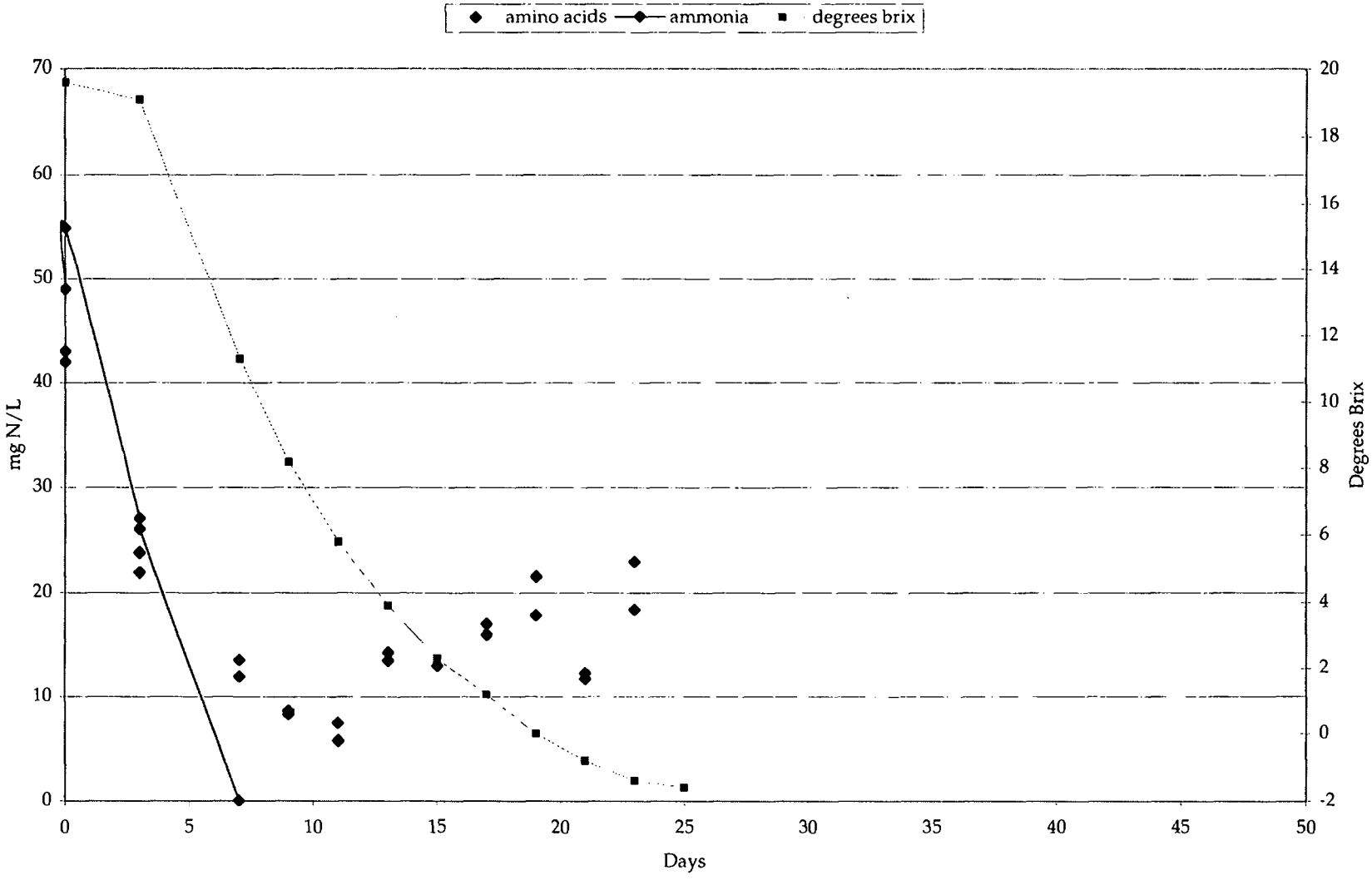

Figure 4.3.8 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 8, DAP 2 lbs/1000 gallons.

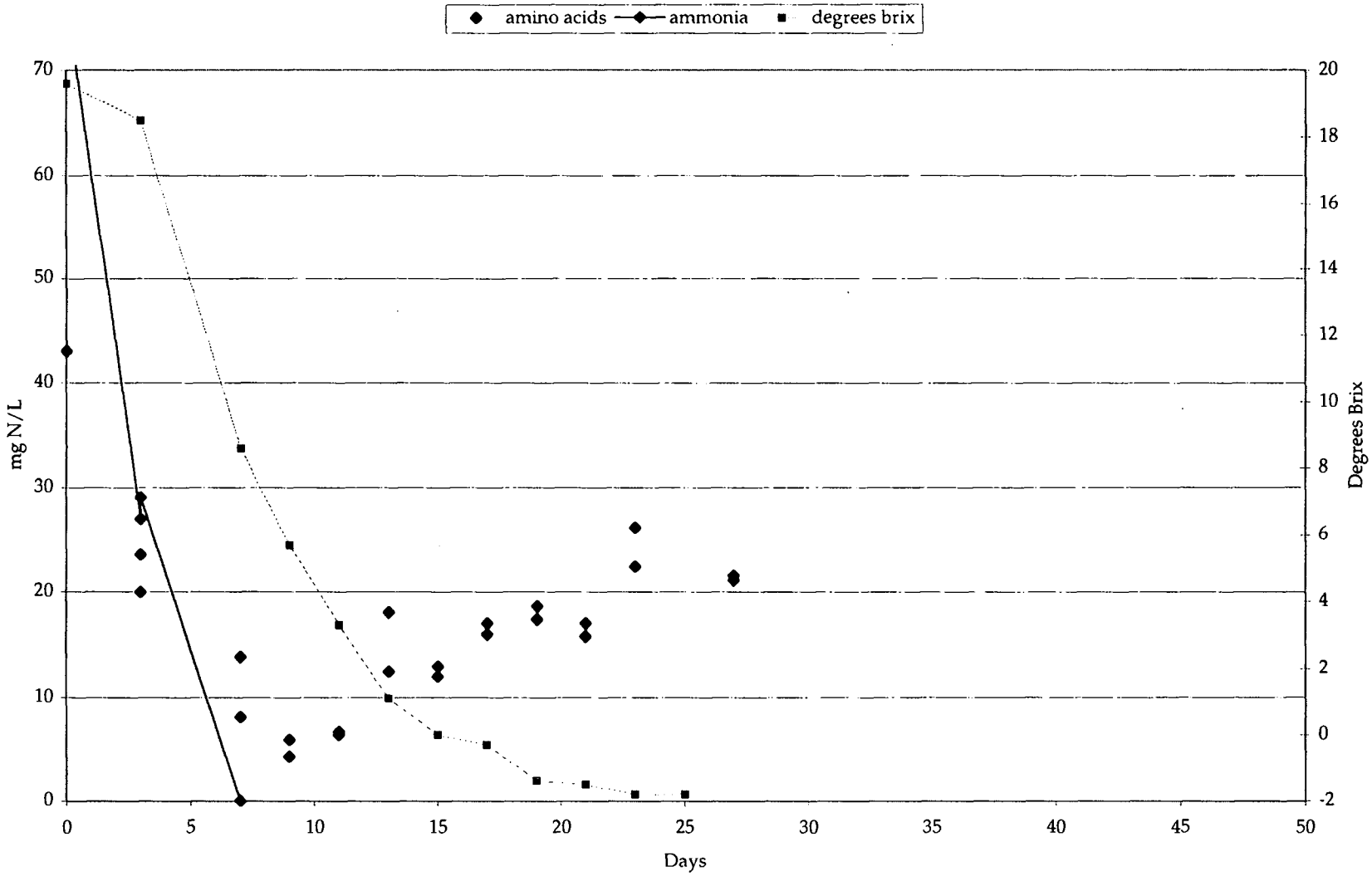

Figure 4.3.9 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 9, DAP 4 lbs/1000 gallons.

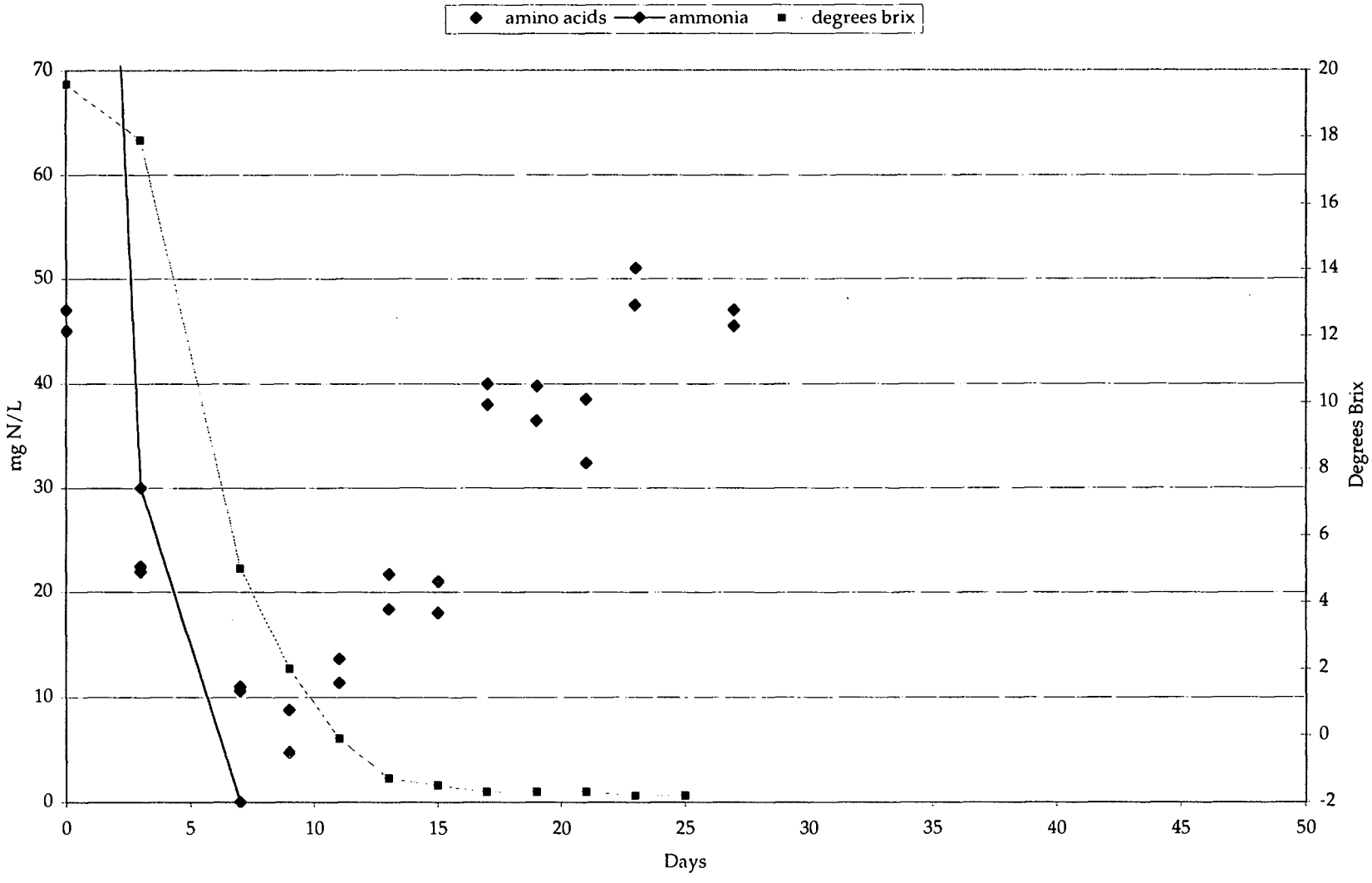

Figure 4.3.10 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 10, DAP 8 lbs/1000 gallons.

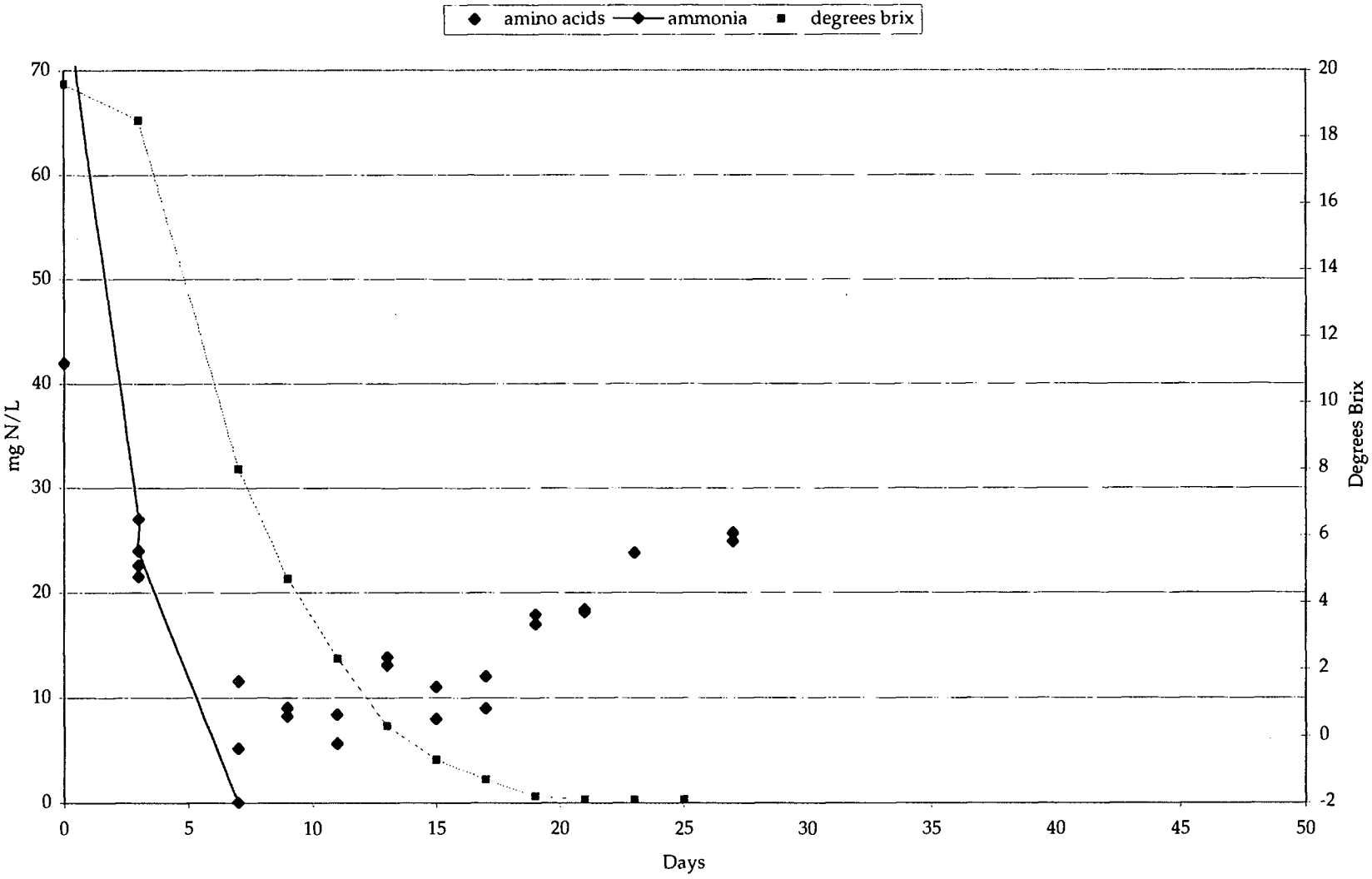

Figure 4.3.11 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 11, DAP 4 lbs/1000 gallons + Yeast Hulls 2 lbs/1000 gallons.

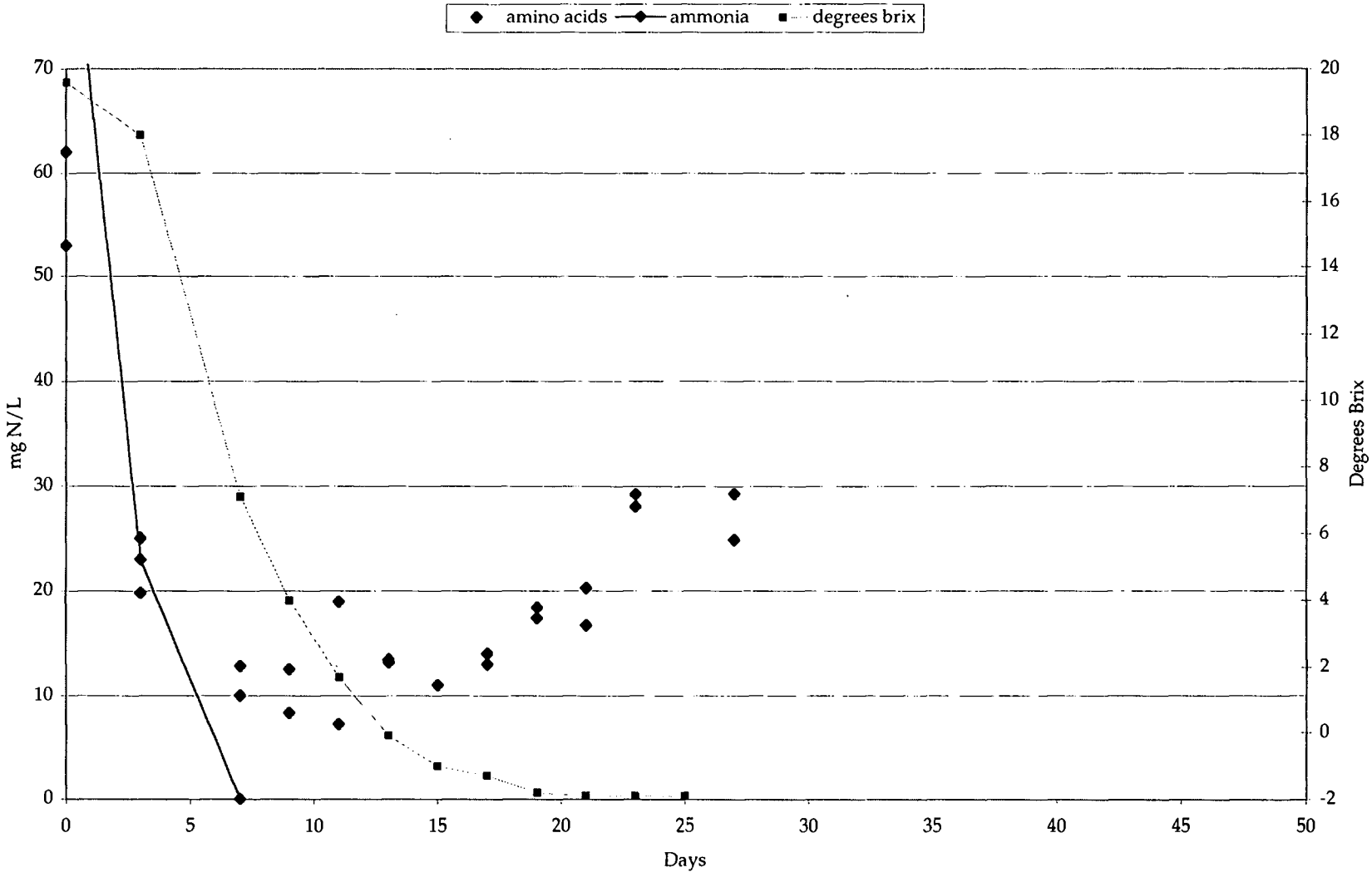

Figure 4.3.12 *Alpha-amino acid and ammonia profiole during fermentation for* Treatment 12, DAP 4 lbs/1000 gallons + PL-50 Yeast Extract 1 lb/1000 gallons.

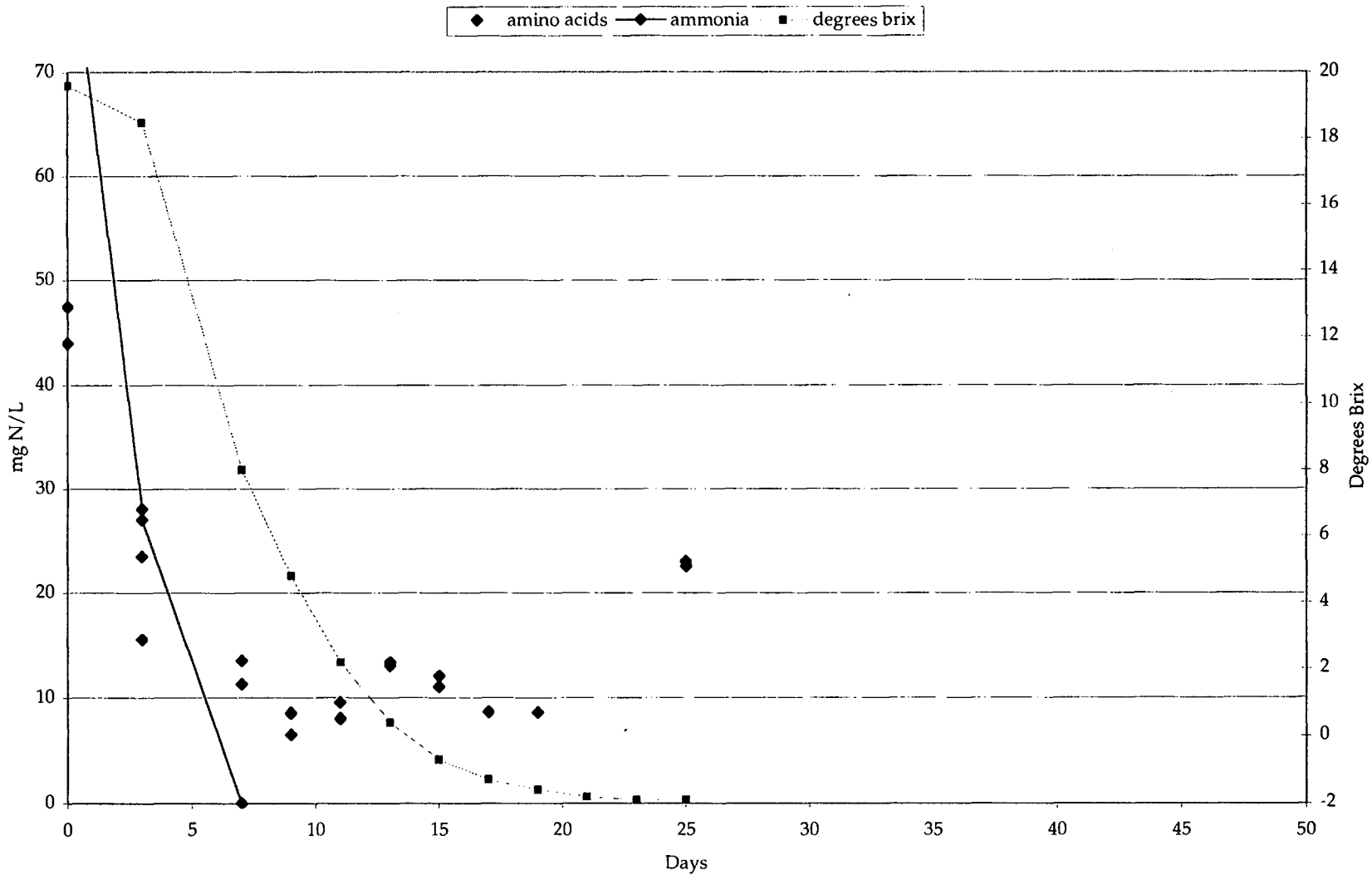

Figure 4.3.13 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 13, DAP 4 lbs/1000 gallons + Thiamine 2 grams/1000 gallons.

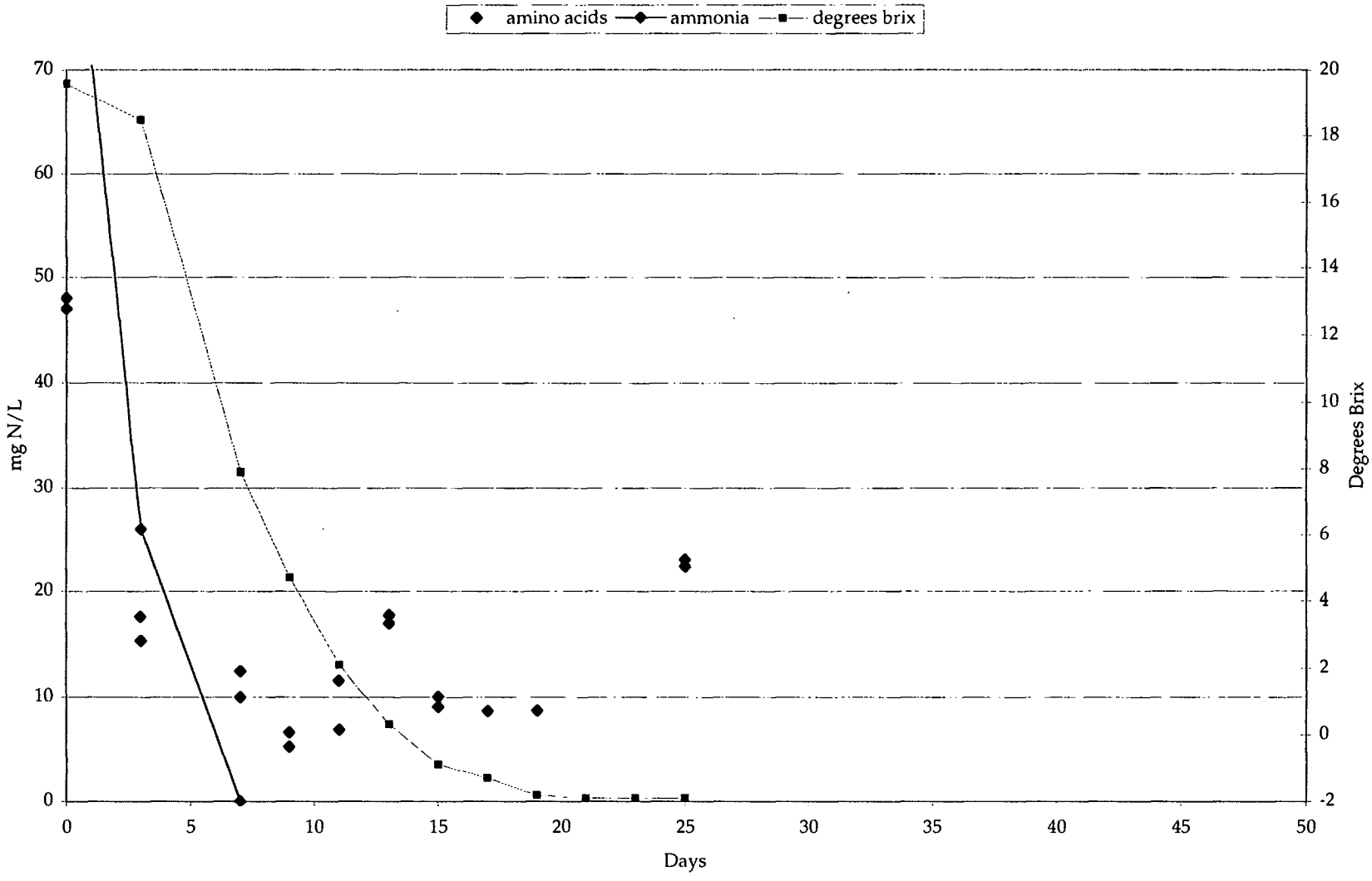

Figure 4.3.14 *Alpha-amino acid and ammonia profile during fermentation for* Treatment 14, DAP 4 lbs/1000 gallons + Thiamine 2 g/1000 + Yeast Hulls 2 lbs/1000 gallons.

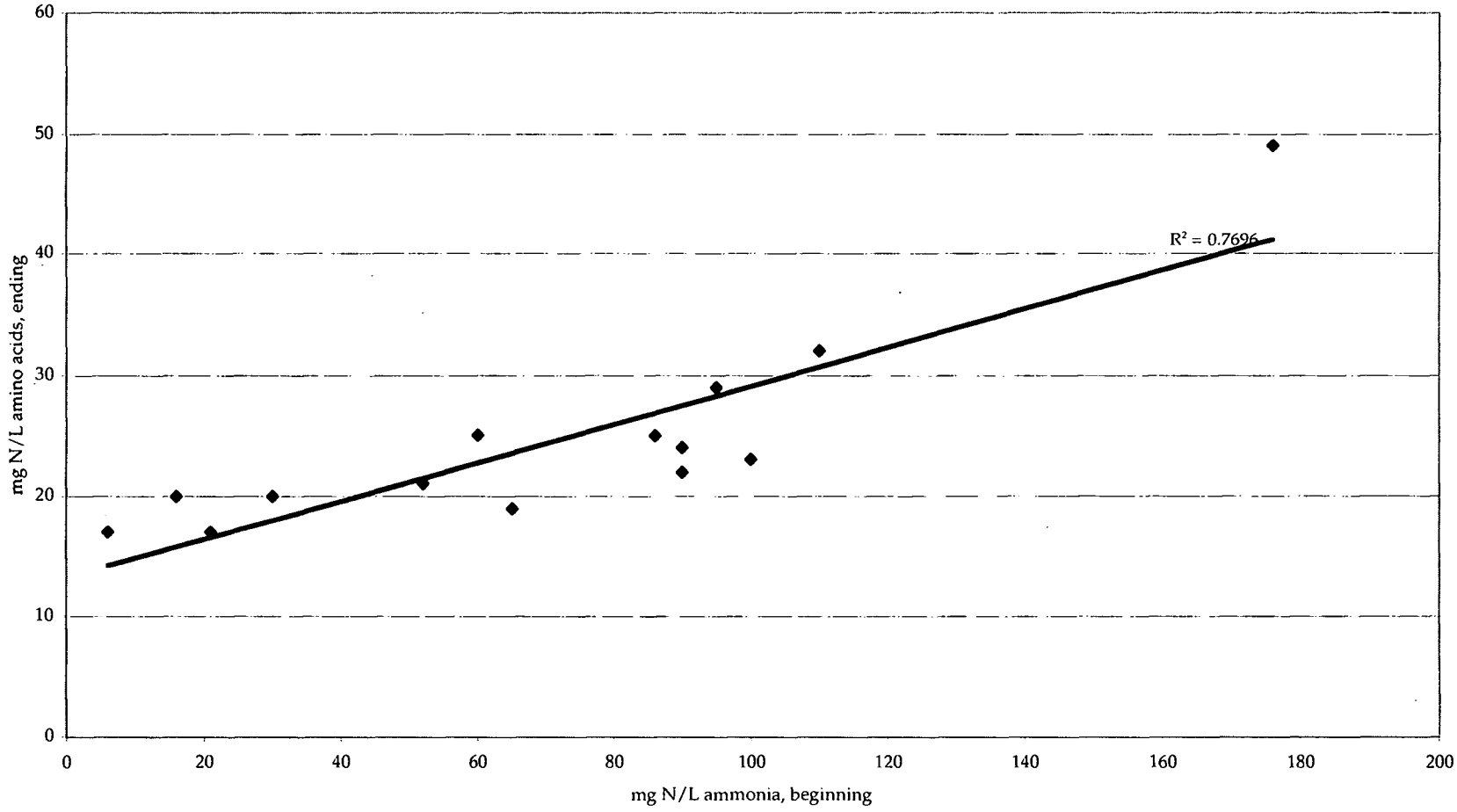

Figure 4.4 *The level of ammonia at the beginning of fermentation is correlated with the level of alpha-amino acids in the new wine.*

4.4 HPLC ANALYSIS OF AMINO ACIDS

4.4.1 CONTROL

The amino acid profiles by HPLC in juice prior to fermentation, mid-way (13° Brix) through fermentation, and at the end of fermentation are shown for the unsupplemented control wines ***(Figure 4.5)***. The amino acids found in the highest concentration in the juice prior to fermentation were proline, arginine, γ-aminobutyric acid and threonine, followed by lower levels of glutamine, phenylalanine, aspartic acid, serine, glutamic acid, alanine, valine, isoleucine and leucine. By mid-fermentation, most of the amino acids, with the exception of proline, had been taken up by the yeast.

4.4.2 COMMERCIAL PREPARATIONS

Superfood at 8 lbs/1000 gallons (100 g/hL) contributed slightly more arginine and, to a lesser degree, aspartic acid, glutamic acid, asparagine, serine, phenylalanine and isoleucine, than the control ***(Figure 4.6)***. Fermaid K at 2 lbs/1000 gallons (25 g/hL), however, did not appear to contain more amino acids than the unsupplemented control juice. The addition of DAP to juice prior to fermentation, especially at the level of 8 lbs/1000 gallons (100 g/hL), produced wines containing higher

concentrations of proline, arginine, alanine, aspartic acid and leucine than the unsupplemented controls *(Figure 4.7)*.

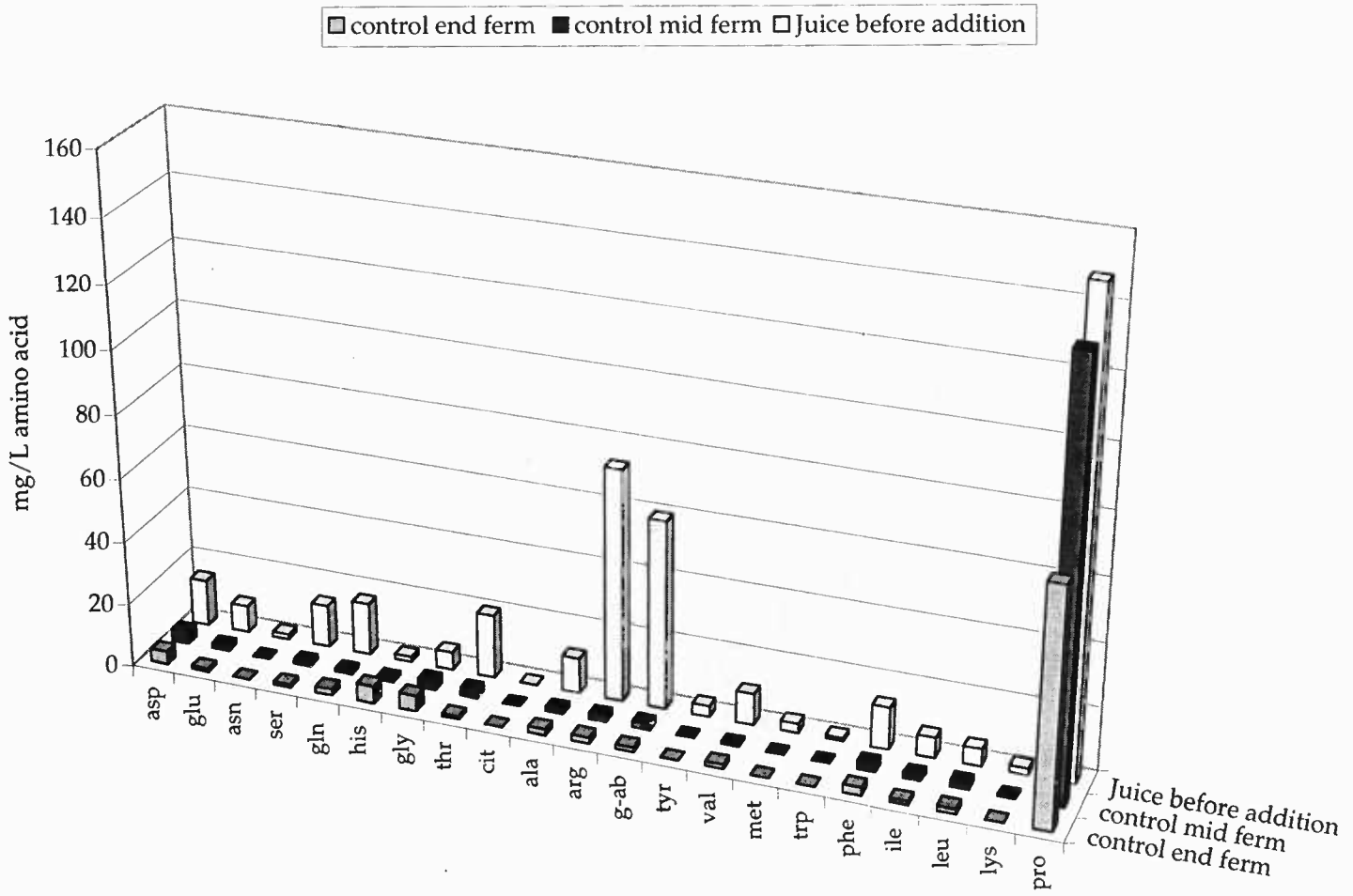

Figure 4.5 *Amino acids by HPLC throughout fermentation for control wine.*

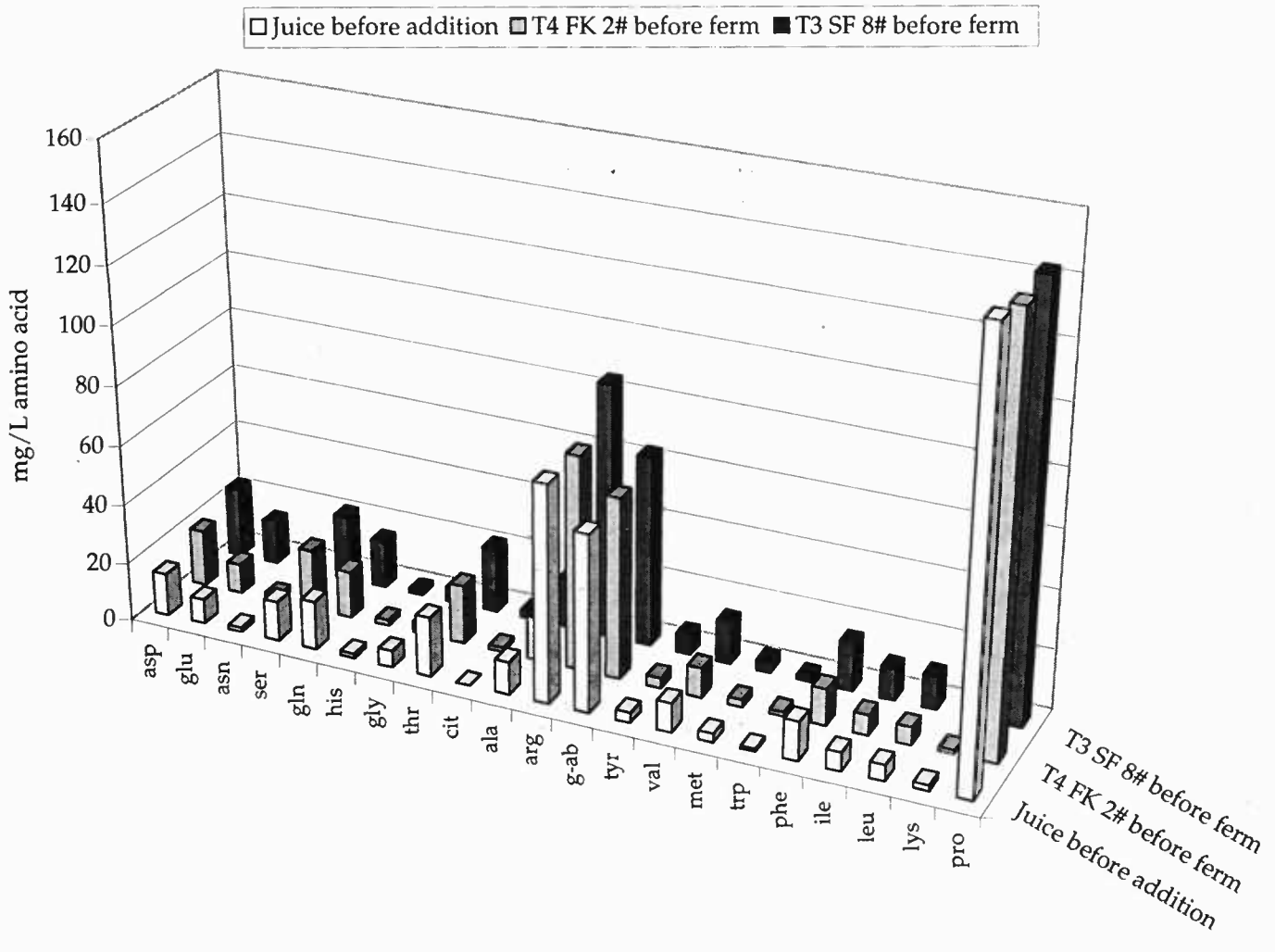

Figure 4.5 *Amino acids by HPLC in Fermaid K and Superfood treated Chardonnay.*

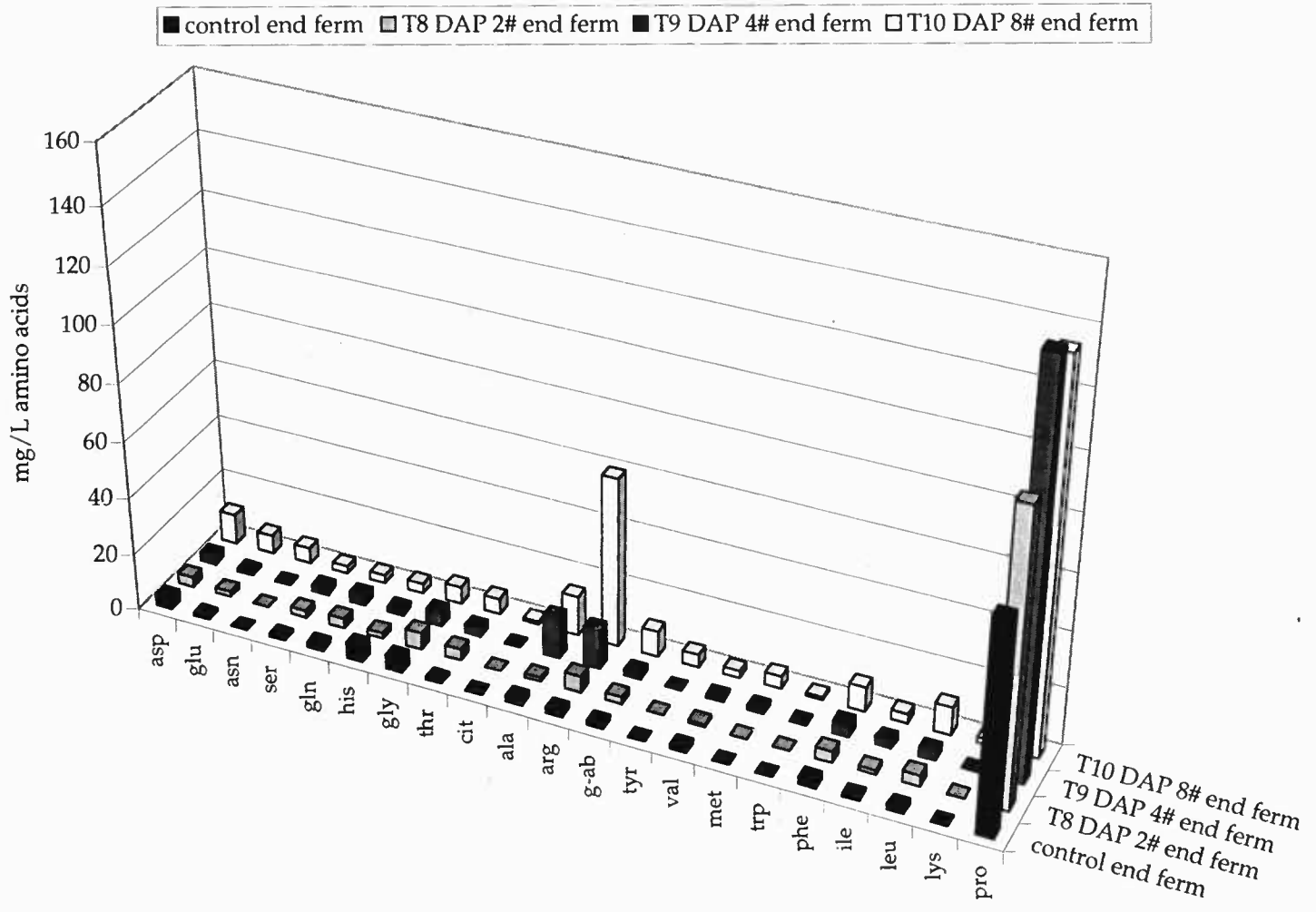

Figure 4.5 *Amino acids by HPLC in DAP treated wines at the end of fermentation.*

4.4.3 Residual amino acids

By the end of fermentation, the proline concentration in the unsupplemented control wines was 50% of the initial juice concentration compared to 70% for the 2 lbs/1000 gallons (25 g/hL) DAP treatment and 90% for the 4 and 8 lbs/1000 gallons (50 and 100 g/hL) DAP treatments *(Figure 4.7)*. The decrease in proline content during fermentation in the unsupplemented control wines could be due to metabolism of proline under conditions of nitrogen deficiency. Enzymes for proline utilization during fermentation are reportedly induced only under conditions of limiting nitrogen (Brandriss and Magasanik, 1979). Salmon and Barre (1998) found that proline was utilized under enological conditions when small amounts of dissolved oxygen were introduced to the fermentation. If dissolved oxygen was present in the extended fermentation of the control, it may explain the apparent proline utilization in this trial.

4.5 GC-MS Analysis

4.5.1 Ethyl carbamate

Due to observed increases in the amino acid content, particularly arginine, in the high DAP supplemented treatments, wine samples were sent to ETS Laboratories, St. Helena, CA, to be analyzed for ethyl carbamate content. Ethyl carbamate, commonly known as urethane, is a known

carcinogen often detected in low concentrations in fermented foods. Detectable levels of ethyl carbamate were less than 2 ng/g (lowest detectable level) in all of the wine treatments. Potential ethyl carbamate analysis, which can estimate potential ethyl carbamate production during aging or exposure to high temperature, was not performed.

4.5.2 SULFIDES

In preparation for a winemaker sensory panel, the new wines were screened for potential aroma and flavor differences (data not shown). 'Reduced' sulfur aromas were detectable in wines from some of the treatments. There were also differences between several duplicate replications, with one replication having detectable reduced character and the other having none. Due to the inconsistencies among some of the replications, these wines were not considered for sensory evaluation of overall aroma and flavor differences.

The wines were analyzed for sulfide profiles using GC-MS by Sara Spayd and David Mee at Washington State University, Prosser ***(Table 4.3)***. These results verified the replication differences found in the sensory screening. Methane thiol (methyl mercaptan) appeared to be above the sensory threshold level (1.5 ppb) for at least one replication of several treatments, including Superfood at 8 lbs/1000 gallons (100 g/hL) and the treatments containing at least 4 lbs/1000 gallons (50 g/hL) of DAP ***(Figure 4.8)***. Several of the replications from treatments were very near the reported sensory

threshold level. Control wines and wines produced from 2 lbs/1000 gallons of DAP (25 g/hL), 4 lbs/1000 gallons (50 g/hL) of Superfood, and 2 lbs/1000 gallons (25 g/hL) of Fermaid K (with and without 2 lbs/1000 gallons (25 g/hL) of DAP) were well below the given sensory threshold levels for all the sulfides, including methane thiol.

Overall, there was a positive correlation between the total sulfide levels present in the wines and the initial juice ammonia concentration prior to fermentation (r^2 = .7127) ***(Figure 4.9)***. This is in contrast to the findings of many previous investigators (Sea *et al.*, 1998; Jiranek *et al.*, 1995; Vos and Gray, 1979.) who found that nitrogen supplementation decreased the development of "reduced character". However, most of the previous work involved juice with YANC levels much higher than those seen in this trial. In any case, previous research does not demonstrate a clear correlation between nitrogen levels and sulfide production (Sea *et al.*, 1998).

Table 4.3 *Sulfide compounds by GC-MS in supplement-treated Chardonnay wines. Bold numbers are above sensory threshold.*

	Hydrogen Sulfide, ppb	Methane Thiol, ppb	Ethane Thiol, ppb	Dimethyl Sulfide, ppb	Diethyl Sulfide, ppb	Dimethyl Disulfide, ppb	Diethyl Disulfide, ppb
sensory threshold, ppb:	~1.0	1.5	1.1 -1.8	17 -25	0.9-1.3	9.8 -10.2	3.6 -4.3
T1R1	0.24	0.57	<lld	3.06	<lld	<lld	<lld
T1F2	0.27	0.66	<lld	3.25	<lld	<lld	<lld
T2R1	0.47	0.89	<lld	3.03	<lld	<lld	<lld
T2R2	0.38	1.03	<lld	3.39	<lld	<lld	<lld
T3R1	0.78	**1.90**	<lld	3.81	<lld	0.29	<lld
T3R2	0.54	**2.13**	<lld	4.28	<lld	0.31	<lld
T4R1	0.40	0.52	<lld	3.55	<lld	<lld	<lld
T4R2	0.66	0.67	<lld	3.95	<lld	<lld	<lld
T5R1	0.46	0.66	<lld	4.39	<lld	<lld	<lld
T5 R2	0.86	0.73	<lld	4.01	<lld	0.19	<lld
T6 R1	0.27	1.00	<lld	4.18	<lld	<lld	<lld
T6 R2	<lld	0.60	<lld	3.18	<lld	<lld	<lld
T7 R1	0.43	**2.00**	0.15	4.30	<lld	0.21	<lld
T7 R2	0.55	**1.90**	0.16	5.00	<lld	0.22	<lld
T8 R1	0.22	0.90	<lld	4.57	<lld	<lld	<lld
T8 R2	0.34	0.97	<lld	5.23	<lld	<lld	<lld
T9 R1	0.80	**1.94**	0.16	5.17	<lld	<lld	<lld
T9 R2	0.25	0.99	<lld	4.12	<lld	<lld	<lld
T10 R1	**1.04**	**2.69**	0.16	5.14	<lld	0.21	<lld
T10 R2	0.58	**2.27**	0.15	4.29	<lld	<lld	<lld
T11 R1	0.15	1.00	0.16	3.95	<lld	<lld	<lld
T11 R2	0.45	**1.88**	<lld	4.38	<lld	0.19	<lld
T12 R1	0.37	**1.69**	0.21	4.48	<lld	<lld	<lld
T12 R2	0.61	**2.29**	0.18	5.57	<lld	0.20	<lld
T13 R1	0.41	**1.53**	0.15	4.67	<lld	0.17	<lld
T13 R2	0.55	**2.03**	0.18	5.47	<lld	0.21	<lld
T14 R1	0.48	**1.51**	<lld	4.26	<lld	<lld	<lld
T14 R2	0.55	**1.92**	0.18	4.98	<lld	0.19	<lld

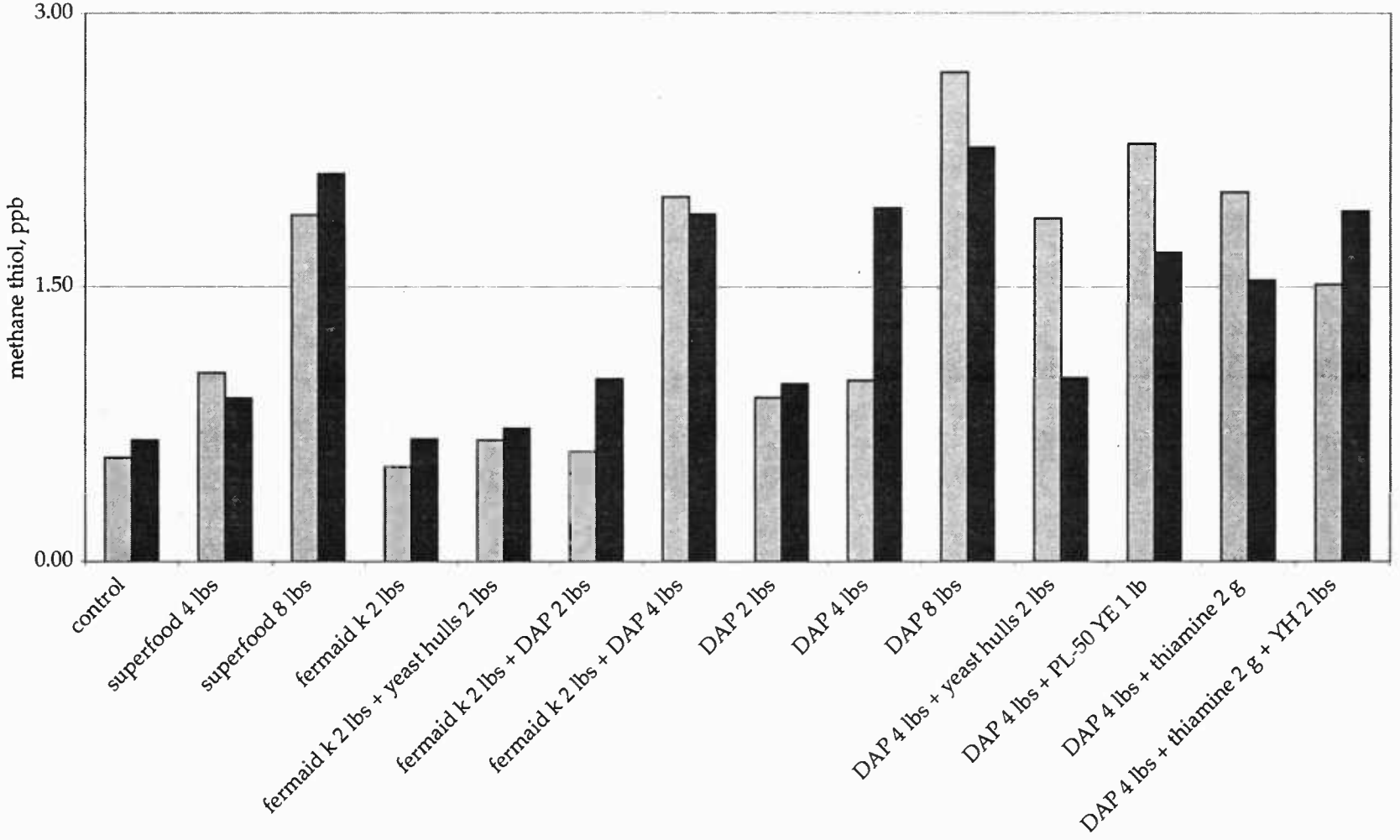

Figure 4.8 *Methane thiol by GC-MS in supplemented wines. At least one replication of several treatments was above the sensory threshold (1.5 ppb).*

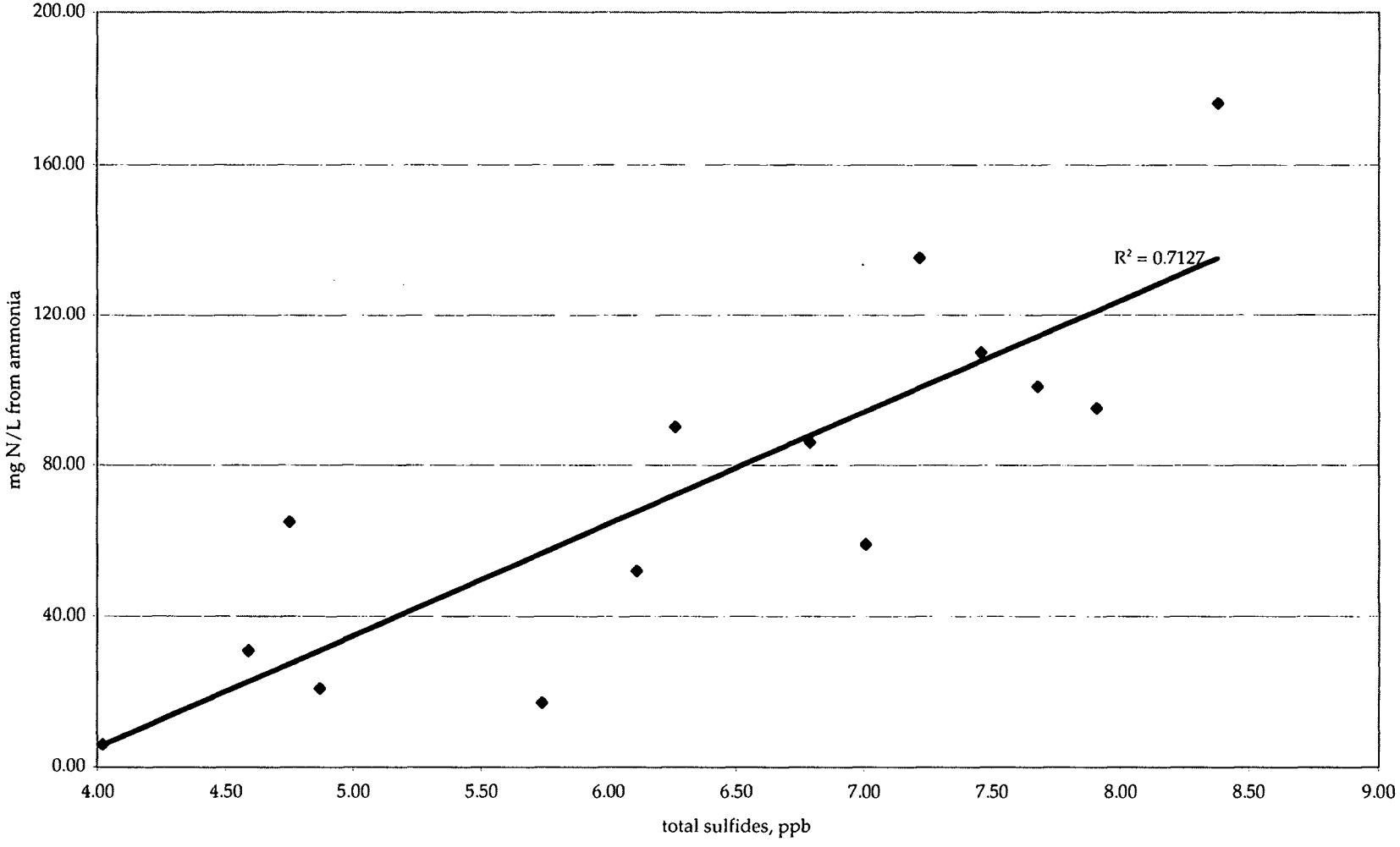

Figure 4.9 *Correlation between initial ammonia level and total sulfides in new wines.*

5. CONCLUSIONS

All treatments increased the rate of fermentation in a nitrogen deficient Chardonnay juice. In general, increases in the yeast assimilable nitrogen content from commercial supplements appeared to be from ammonia and not from amino acids. This is supported by both the NOPA data and the HPLC amino acid analysis.

By comparing the rate of fermentation with the initial YANC level, there is a dramatic increase in fermentation rate with the mid-level treatments, including Treatments 2, 4, 5, 6, 7 and 9. This increase in fermentation rate appears to reach a maximum at mid-level additions (those approaching 150 mg N/L); high levels of DAP did not ferment much faster than the mid-level treatments. This is in agreement with the findings of Bezenger and Navarro (1987), who observed maximum fermentation rates at a mid-level range of available nitrogen, with decreased rates both above and below.

Ammonium ion was quickly depleted during fermentation and was not present in the finished wine. Although amino acids were also depleted, several treatments had relatively high concentrations of amino acids at the end of fermentation. A direct relationship was found between the initial ammonia level and the amino acid concentration at the end of fermentation.

Sulfide compounds, including methane thiol (methyl mercaptan), were above sensory threshold levels in several treatments supplemented with high amounts of nitrogen, while they were below threshold in the treatments with lower nitrogen supplementation. In general there was a direct relationship between YANC level and sulfide level.

Because cell densities are not known, the increase in amino acid and sulfide compounds at the end of fermentation could be due to a generally higher cell number in the more vigorous fermentations. Yeast autolysis, while a possible source of amino acid and sulfur compounds, was probably not responsible for these results because the wine had not been in contact with the yeast lees for an extended period of time.

Because of the importance of yeast strain in fermentation behavior, future work should address yeast strain differences. In addition, it would be useful to monitor cell density, as this variable may affect the level of all components in the finished wine. Ethyl carbamate compounds generally form in the presence of heat; because of this it may be more useful to assay samples for potential ethyl carbamate compounds, rather than ethyl carbamate, which may not have developed in the new wine.

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