## Technical Brief

# Comparison of Analytical Methods for Prediction of Prefermentation Nutritional Status of Grape Juice

Barry H. Gump,[1] Bruce W. Zoecklein,[2]* Kenneth C. Fugelsang,[1] and Robert S. Whiton[3]

Five methods for evaluating nitrogen status were compared using 70 Cabernet Sauvignon juice samples: nitrogen by *o*-phthaldialdehyde (NOPA), arginine NOPA, enzymatic ammonia, Formol, and high-performance liquid chromatography (HPLC). Parallel recovery studies using model solutions of various amino acids and ammonia, presented singly and in combination, were also conducted. The results from two fruit-processing methods were compared using immature and mature berries. NOPA measurements were significantly higher in mature, pressed whole berry-derived samples, compared with homogenized juice. Adjustment of formaldehyde pH prior to analysis was found to be critical to consistency of the Formol method. Average amino acid recoveries for the Formol titration ranged from 82 to 99%. Average recovery for proline was 16.9 ± 0.4%. Ammonium nitrogen was also recovered (84 ± 3%) in the Formol procedure. Formol results trended significantly with NOPA. The correlation coefficient between Formol and NOPA plus $NH_4^+$ was 0.87, with Formol values being higher. The average deviation between the Formol and HPLC plus $NH_4^+$ and between the NOPA plus $NH_4^+$ and HPLC plus $NH_4^+$ was 7.3%.

*Key words:* Assimilable nitrogen, Formol, HPLC, NOPA, FAN, YANC, amino acids, $NH_4^+$

Rapid, accurate, and precise analytical methods for assimilable nitrogen involving simple sample preparation, minimal waste products, and minimal instrumentation would be valuable tools for winemakers. Currently, several analytical methods are in common use. The Formol titration is a simple and rapid determination of assimilable nitrogen. This method involves the addition of neutralized formaldehyde to liberate protons that are titrated directly with NaOH to a pH 8.0 end point [12]. The analysis provides an approximate, but useful, index of the nutritional status of juice. The more recent nitrogen by *o*-phthaldialdehyde (NOPA) procedure [3] has been used to obtain free α-amino acid nitrogen (FAN) by derivitization of primary amino groups with *o*-phthaldialdehyde (OPA). The resulting isoindole derivatives can be measured spectrophotometrically at 335 nm. As a result of the specificity of this reaction to primary amino acids, the imino acid proline cannot form a derivative and, therefore, does not contribute to the results. Ammonium ion, the second main source of assimilable nitrogen, is not measured by the NOPA procedure. A modification of NOPA, ARGOPA [1] allows for selective determination of arginine, quantitatively the most significant contributor to yeast nutrition and potential ethyl carbamate formation. This procedure uses cation exchange to isolate arginine from other amino acids prior to analysis by NOPA.

HPLC combines speed and sensitivity in quantifying individual amino acids. It is achieved with automated, online derivatization, resulting in a rapid, accurate, sensitive, and reproducible analysis of amino acids [5].

This investigation compared a modified Formol titration [4], NOPA, ARGOPA, enzymatic ammonia, and HPLC methodologies for analysis of FAN and yeast assimilable nitrogen compounds (YANC).

## Materials and Methods

All juice analyses were conducted using fruit from mature (10-year-old) Cabernet Sauvignon (*Vitis vinifera* L.) vines grown in northwestern Virginia. Samples (50 berries from each side of the canopy) were randomly collected from five replicates of three vine plots in a completely randomized design. During the 1999 harvest season, berries were collected five times during fruit maturation, on 30 August, and 4, 11, 18, and 27 October, and stored at -25°C for subsequent analysis.

**Sample preparation**. Juice produced by two fruit-processing methods was compared using immature (16.6 Brix) and mature (21.5 Brix) fruit. Fruit samples stored at -25°C were warmed to 10°C before processing. Processing methods were pressed whole berries, using a Tekmar Laboratory Stomacher® (model 400; Cincinnati, OH) for 2 min, and whole berry homogenates, using a Waring Commercial Laboratory® blender

[1]Professors, Department of Viticulture and Enology, California State University, Fresno, CA 93740; [2]Associate Professor and Head, and [3]Research Scientist, Enology-Grape Chemistry Group, Department of Food Science and Technology, Virginia Tech, Blacksburg, VA 24061.

*Corresponding author [Fax: 540 231-9293; email: bzoeckle@vt.edu]

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(model 7010; New Hartford, CT) for two pulses of 2-sec duration at the low setting. Seeds were carefully examined with each procedure to ensure no breakage. All subsequent analyses were conducted on fruit samples prepared by whole berry homogenation.

**Analysis of juice nitrogen components**. The Formol titration procedure used was that described by Ogorodnik and Merkureua [12], modified by Gump et al. [4], and further modified and posted on the Enology-Grape Chemistry web site (http://www.fst.vt.edu/zoecklein/index.html or http://www.vtwines.info). Formaldehyde readily combines with the free amino groups to produce methylol derivatives. The reaction causes an isoelectric amino acid to lose a proton from the $NH_3$ group of the zwitterion form. The liberated proton can be titrated directly with NaOH to a pH 8.0 end point. Results can be calculated using the general equation: mg nitrogen/L = [(vol NaOH) x (concn NaOH) x 14 x (dilution factor) x 1000]/(sample vol).

The nitrogen by OPA (NOPA) procedure [3] involves derivatization of primary amino groups with *o*-phthaldialdehyde. The resulting isoindole derivatives were measured at 335 nm, using a Genesys 5® spectrophotometer (Spectronic Instruments, Rochester, NY). Ammonia was determined enzymatically as described by Boehringer Mannheim (1995; Indianapolis, IN). This procedure reports results in terms of mg nitrogen/L.

The arginine concentration in the juice was determined by a modification of the NOPA procedure (ARGOPA) as described by Butzke and Austin [1]. The determination uses cation exchange to isolate arginine from other amino acids. In contrast to the NOPA procedure, results are reported in mg arginine/L. To convert to mg nitrogen/L, the results are multiplied by the factor 0.16 (assuming two assimilable nitrogens from arginine).

HPLC analysis was conducted using an Agilent 1100 HPLC system (Palo Alto, CA) and a Xterra MS C18®, 5 µm, 3.9 x 150 mm column (Waters, Milford, MA). The procedure used automated, online derivatization with *o*-phthaldialdehyde for primary amino acids, and 9-fluorenylmethyl chloroformate (FMOC-CL) for secondary amino acids, as described by Henderson et al. [5].

## Results and Discussion

**Sample preparation**. Grape tissue type (pulp versus skin) and fruit maturity have been shown to influence nitrogen concentration [2,7,8,9,15]. Given the importance of assimilable nitrogen, it is essential that processing methods be standardized. The results from two fruit-processing methods were compared using both immature (16.6 Brix) and mature (21.5 Brix) Cabernet Sauvignon berries. NOPA and Formol analyses of juice nitrogen from pressed whole berries (Stomacher) and juice homogenate (blender) were compared (Figure 1). Results of the Formol analysis were not significantly influenced by the sample processing method. However, the NOPA measurements were significantly higher in the Stomacher, compared with the juice homogenate, from mature fruit (average differences, 21.6 mg/L, n = 12). Presumably, the berry homogenates provide a maximum extraction of the amino acids from both pulp and skins, whereas Stomacher processing extracts amino acids mainly from the pulp. It has been reported that the nitrogen is localized mainly in the solid portion of the grape clusters [2,7,8,9]. Stines et al. [15] determined that 15% of the total amino acids were found in the skins and 77% in the pulp of mature Cabernet Sauvignon. This is roughly proportional to the percentage by weight of these fruit components. The primary amino acid levels reported by Stines et al. [15] were approximately four times higher in the pulp than in the skins. Therefore, the skin/pulp homogenate mixtures would be diluted and contain lower concentrations of these amino acids, leading to lower NOPA measurements. The ratio of arginine/proline was about 1.5:1 in immature fruit and increased to about 7:1 in mature grapes. The small differences in nitrogen concentration between the two sampling methods, as noted in immature fruit, may have been the result of the arginine/proline ratio. A number of studies [7,8,9,10,14] have shown that the degree of berry maturity has a strong influence on the amino acid composition. The Formol method titrates primary amino acids, $NH_4^+$ ions, and about 17% of the available proline (see below). The immature fruit, containing a lower concentration of proline and ammonia, would be expected to parallel the NOPA analysis.

**Formol pH.** Adjustment of formaldehyde pH before analysis was critical to the reproducibility of the Formol method. Several experiments were conducted involving the recovery of proline, using the Formol titration (Table 1). The use of non-pH–adjusted formaldehyde (pH 2.75) resulted in a significant difference between calculated and recovered nitrogen (Table 1, columns 2 and 3, respectively). The results of adjusting the formaldehyde to pH 8.0 before analysis are shown in column 4. These data indicate that unadjusted formaldehyde, by itself,

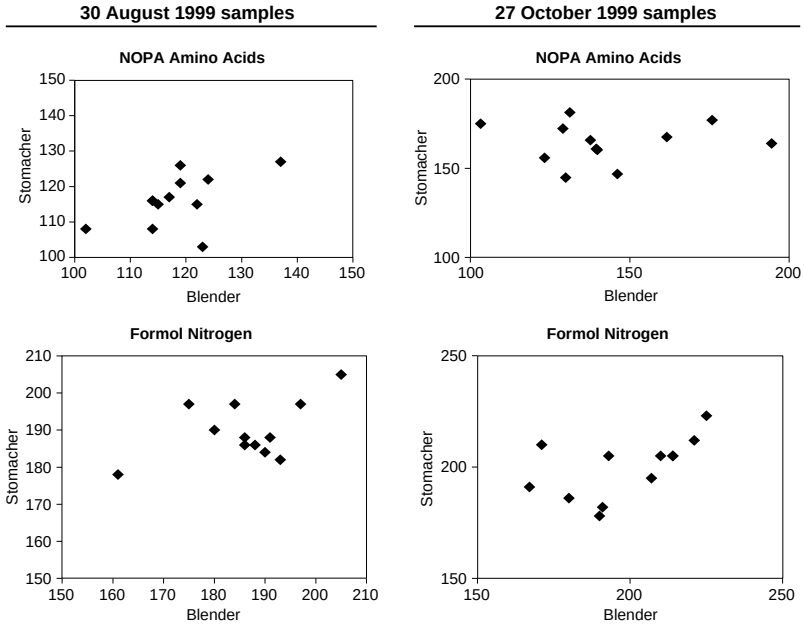


**Figure 1** Effect of fruit-processing method (Stomacher and blender) on Cabernet Sauvignon fermentable nitrogen by Formol titration and NOPA (two sample dates).

**Table 1** Recovery of proline and effect of formaldehyde pH on Formol titration.

| Proline standard (mg/L) | Calculated nitrogen (mg/L) | Recovered nitrogen (mg/L) | Nitrogen found with pH 8 formaldehyde (mg/L) | Nitrogen due to titration of pH 8 formaldehyde (mg/L) | Recovery of proline nitrogen (%) |
|---|---|---|---|---|---|
| 0 | 0 | 151.6 | 0 | 151.6 | — |
| 200 | 24.3 | 154.0 | 4.1 | 149.9 | 16.9 |
| 600 | 73.0 | 162.3 | 11.5 | 150.8 | 17.1 |
| 1000 | 121.6 | 170.4 | 21.7 | 148.7 | 17.0 |
| 1400 | 170.3 | 175.8 | 28.2 | 147.6 | 16.6 |
| 1800 | 219.0 | 191.8 | 38.9 | 152.9 | 17.8 |

was being titrated in amounts equivalent of approximately 150 mg nitrogen/L. Low formaldehyde pH resulted in significant overtitration and high apparent values of fermentable nitrogen. The authors have noted that new bottles of formaldehyde have initial pH values near 3.5 and that they can degrade further by generating acid, requiring pH adjustments every few days. As noted in Table 1, proline nitrogen was partially titrated in the Formol procedure to a level of approximately 17%.

**Formol recovery.** In a separate set of experiments, a series of amino acid standards and ammonium chloride were titrated using the Formol procedure (Table 2). These data suggest that while there is some variability in recovery of individual amino acids, mixtures of amino acids were recovered to approximately 85%. If it is assumed that arginine has one titratable nitrogen, then it is also titrated to the same extent.

The addition of ammonium ion to the mixture listed in Table 2 showed an increase in mg nitrogen/L equal to the nitrogen added, indicating that the recovery was essentially quantitative.

**Table 2** Recovery of amino acid standards and ammonium ions by Formol titration.

| | Added amount (mg/L) | No. of trials | Nitrogen in solution (mg/L) | Nitrogen by Formol (mg /L) | Avg nitrogen found (%) | SD (%) |
|---|---|---|---|---|---|---|
| L-Alanine | 99.4 | 4 | 15.6 | 15.4 | 96.4 | 4.5 |
| Previous mixture plus γ-amino butyric acid | 58.5 | 4 | 23.56 | 21.0 | 89.2 | 0.03 |
| Previous mixture plus L-arginine[a] | 113.4 | 3 | 32.67 | 28.0 | 85.7 | 0.00 |
| Previous mixture plus L-aspartic acid | 36.0 | 3 | 36.46 | 30.8 | 84.5 | 0.00 |
| Previous mixture plus L-glutamic acid | 46.2 | 3 | 40.86 | 33.6 | 82.2 | 0.00 |
| Previous mixture plus L-serine | 90.1 | 3 | 52.86 | 43.4 | 82.1 | 0.00 |
| Previous mixture plus DL-threonine | 59.0 | 3 | 59.79 | 50.4 | 84.3 | 0.00 |
| Previous mixture plus ammonium chloride | 200.0 | 3 | 112.14 | 96.6 | 86.1 | 0.00 |
| Previous mixture plus L-proline | 1072.9 | 4 | 242.64 | 119 | 48.9 | 0.28 |

[a]Accounts for only one nitrogen in arginine.

With the addition of proline, overall recovery decreased to less than 50%. While the addition of proline produced a theoretical nitrogen increase of 130.5 mg/L, only 22.4 mg/L of this amount was actually recovered. Thus, proline is only partially titrated, with an average recovery of 16.9%.

In a separate series of trials, results of the Formol titrations were compared for samples (n = 6) that were allowed to sit for one hour at pH 8.0 before being treated with formaldehyde and titrated versus samples that were neutralized and immediately treated with formaldehyde and titrated (n = 5). Average titration volumes differed by only 0.01 mL for the two series (data not shown). Therefore, the delay had no significant impact on the results. In a second set, the results for samples (n = 10) titrated rapidly to the end point were compared with those tritrated slowly and with considerable mixing prior to each addition of base (titration time of 4 to 5 min, n = 4). While the delayed titration increased the Formol results by an average of 19.4 mg/L nitrogen, the differences were not statistically significant at $p \leq 0.05$ (data not shown). Both of these trials suggest the robust nature of the Formol titration method and its insensitivity to common operational variations. Additionally, titration of clarified juice samples containing up to 50 mg/L total sulfur dioxide had no influence on the Formol results (data not shown).

**Formol minimization**. Commercial formaldehyde is 37% formaldehyde wt/wt (40% wt/vol), 10 to 15% methanol, with water as the balance. As a result, formaldehyde is flammable, an inhalation, skin, and eye irritant, and a possible carcinogen, and it is preferable to use the smallest quantity possible of this hazardous material. A series of experiments was conducted to determine the potential for reducing the volume of this reagent required by reducing the sample volume titrated. A white wine was titrated using three different sample volumes, with a proportional reduction in formaldehyde (Table 3). The average results obtained using the three different sample volumes did not differ statistically. These results indicate that equally quantitative results can be obtained by reduced volume titrations. Currently, these titrations are routinely being conducted with 4-mL samples and 1.25 mL formaldehyde (http://www.fst.vt.edu/zoecklein/index.html or http://www.vtwines.info).

**Methods correlations**. Formol results were positively correlated with NOPA plus ammonia by enzymatic analysis with a correlation coefficient of 0.87 (n = 87). Shively and Henick-Kling [13] reported a correlation coefficient of 0.97 for NOPA (in the absence of ammonia analysis) and the Formol method and a notable agreement between the results obtained by the two methods. These results are not consistent with the current

study. The Formol values in the current study were consistently higher (Figure 2), as expected for two reasons. We have found that the Formol method titrates 77 to 88% of the $NH_4^+$. This is considerably higher than the 3.5% response to ammonium ions reported in the NOPA procedure [3]. Additionally, we have noted that 17% of the proline concentration is titrated with the Formol procedure. Results of the current study consistently demonstrate that the Formol analysis method results in a 28.5% higher nitrogen value than the NOPA procedure and 18.4% higher values than NOPA plus ammonia. Both the NOPA and Formol methods measure/titrate only one of arginine's four nitrogens. Since arginine hydrolyzes to ornithine and urea, providing two assimilable nitrogens to the yeast [6], both methods understate the arginine content of a juice sample. The Formol method compensates for this by partially titrating proline.

HLPC-derived assimilable nitrogen concentrations were determined by adding the nitrogen contributions from each of the assimilable amino acids listed in Table 4. As seen in Figure 2, the Formol results trended significantly with NOPA. The correlation between Formol and NOPA plus $NH_4^+$ was 0.87, with the Formol values being generally higher. The average difference between the Formol and HPLC plus $NH_4^+$ data was 7.3% (n = 75). The average difference between the NOPA plus $NH_4^+$ and HPLC plus $NH_4^+$ was -7.3% (n = 78) (Figure 2). This is inconsistent with the results of Mee et al. [11], who reported that NOPA gave an average value 5% higher than the HPLC values for selected assimilable amino acids. Our HPLC results are higher because we considered arginine to provide two nitrogen, while the NOPA only counts one.

The Formol nitrogen values correlated positively with the HPLC analysis of arginine (correlation coefficient of 0.78). An examination of the NOPA data indicated a strong correlation with HPLC-derived arginine concentrations (correlation coefficient 0.81). In a comparison of 22 samples run by ARGOPA and HPLC, we obtained good agreement between results from the two methods (average difference between values of 1.0 mg/L, correlation coefficient of 0.88). An examination of nitrogen status of Chardonnay fruit showed trends similar to those of Cabernet Sauvignon (data not shown).

## Conclusions

NOPA recovers only ~3.5% of the ammonia in a typical grape sample [3], so separate ammonia analyses must be run to obtain fermentable nitrogen values. The Formol method titrates ammonia together with amino acids. Formol and NOPA understate arginine (by titrating/reacting only one assimilable nitrogen). Formol also undertitrates other amino acids (~85% recovery), but partially compensates by overstating the nutritional status by the amount of proline titrated (~17% recovery). There is a positive correlation between NOPA and the Formol titration method, which adds confidence to both analytical procedures. While the numbers obtained may differ somewhat, both NOPA and Formol provide rapid and useful estimations of the nutritional status of juice. An examination of nitrogen status of Chardonnay fruit showed trends similar to those of the Cabernet Sauvignon grapes (data not shown).

From this investigation, it can be concluded that all methods evaluated can provide valuable information to the winemaker and grower. The Formol and NOPA plus ammonia

**Table 3** Comparisons of reduced volume Formol titrations.

| Sample | Nitrogen mg/L | Avg ± SD |
|---|---|---|
| 25.0 mL + 6.25 mL formaldehyde | 288, 302, 325 | 305 ± 18 |
| 10.0 mL + 2.5 mL formaldehyde | 298, 294, 353 | 315 ± 32 |
| 5.0 mL + 1.25 mL formaldehyde | 280, 294, 322, 308 | 301 ± 18 |

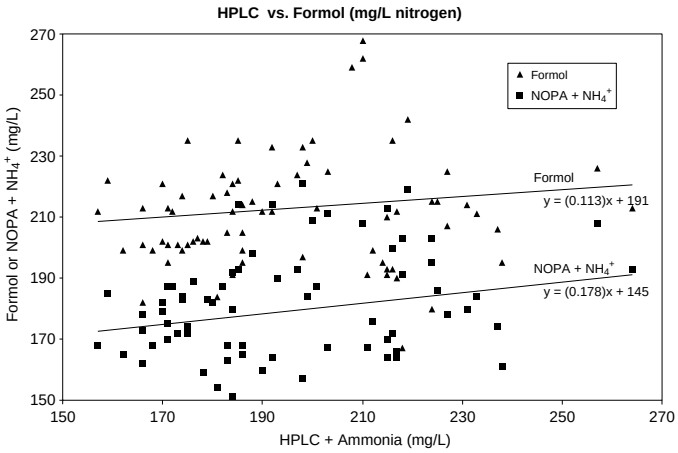


**Figure 2** Correlation between Formol and HPLC plus $NH_4^+$ and NOPA plus $NH_4^+$ and HPLC plus $NH_4^+$ .

**Table 4** HPLC-derived assimilable nitrogen concentrations in Cabernet Sauvignon juice.

| | Nitrogen concn (mg/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample | Aspartic acid | Glutamic acid | Serine | Threonine | Arginine | Methionine | Proline | Sum |
| 1 | 1.5 | 2.7 | 7.4 | 6.5 | 75.7 | 1.2 | 53.9 | 149.1 |
| 2 | 1.6 | 2.5 | 8.0 | 6.9 | 85.1 | 1.3 | 60.0 | 165.6 |
| 3 | 1.7 | 2.7 | 6.6 | 6.4 | 85.5 | 1.2 | 50.3 | 154.3 |
| 4 | 1.5 | 2.1 | 7.1 | 6.4 | 79.9 | 1.1 | 59.0 | 157.1 |
| 5 | 1.9 | 3.7 | 8.1 | 7.2 | 76.2 | 1.2 | 73.0 | 171.4 |
| 6 | 1.6 | 3.5 | 8.3 | 7.2 | 98.2 | 1.4 | 60.3 | 180.6 |
| 7 | 1.6 | 3.6 | 7.9 | 6.7 | 71.3 | 1.3 | 45.1 | 137.5 |
| 8 | 1.5 | 3.4 | 8.1 | 7.5 | 84.4 | 1.3 | 87.4 | 193.7 |
| 9 | 1.8 | 2.8 | 7.3 | 6.2 | 70.0 | 0.9 | 63.8 | 152.8 |
| 10 | 2.0 | 2.8 | 6.9 | 5.9 | 55.9 | 1.0 | 84.4 | 158.8 |
| 11 | 1.4 | 3.1 | 7.2 | 6.0 | 56.7 | 1.1 | 88.4 | 163.9 |
| 12 | 1.9 | 2.6 | 8.7 | 7.7 | 103.0 | 1.3 | 71.5 | 196.8 |

methods give values representing the general overall nutritional status of juice and wine. As such, the method used can be chosen for the convenience of the analyst. The arginine by NOPA and HPLC methods provide measurements for arginine and other specific amino acids, respectively. As a result of its precursor role, arginine is of importance in ethyl carbamate formation. The ability of the HPLC method to provide measures of the entire suite of amino acids allows a better understanding of yeast fermentation dynamics and the impacts of vineyard and processing practices on grape amino acid composition. Studies are currently underway to evaluate these impacts.

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