From: Method by Dr. Christian Butzke, U.C. Davis. The reagents have been changed slightly for better shelf life. In the original method, NaOH, boric acid, NAC, and OPA were combined, but this solution is stable for only a few days. By keeping the reagents separate until use, they last much longer.

BACKGROUND

Nitrogen is the primary limiting element in the growth of yeast cells. Wine fermentations will go more quickly and have less problems going to completion if there are sufficient amounts of nitrogen present in the initial juice.

Nitrogen is present in grape juice primarily as ammonia and amino nitrogen. Ammonia is the simplest form of nitrogen in the grape and is a result of cellular breakdown reactions. Amino-nitrogen is the nitrogen present in amino acids, produced in the cells of the berry to allow cell growth and function. The amounts of ammonia and amino-nitrogen are independent of each other since they come from different sources and serve different purposes in the cell.

Yeast use nitrogen for building more yeast cells, as well as for production of enzymes and other proteins, including sugar transport proteins, during fermentation. In the initial stages of wine fermentation yeast will absorb, the simpler, ammonia as a source of nitrogen for growth. Very shortly the yeast will begin to use the more complex amino-nitrogen. When fermentation is well underway most of the amino acids are used up by yeast growth and have been absorbed into the yeast cells.

Proline is an amino acid that is not used by Saccharomyces yeast because oxygen is needed to break it down, but oxygen is not present during active fermentation. A measure of all amino acids would include proline, which can be over 50% of the amino acid content, thus overestimating the amount of nitrogen available to the yeast.

The measurement of alpha amino nitrogen by OPA is a dye-binding test measuring the amino acids except proline. This makes it an accurate measure of the truly yeast available amino nitrogen. By measuring the ammonia level and amino nitrogen level by OPA (NOPA) an accurate view of the nitrogen status of the juice is presented. By knowing the nitrogen status of a juice, more accurate adjustments can be made to supplement the juice appropriately.

Additions of ammonia, as diammonium phosphate (DAP), and of complex amino acids (legally added as yeast extract), can now be balanced to optimize the initial nitrogen content of a particular grape juice before fermentation.

Ammonia is measured by an enzyme test. The amino nitrogen is measured by the NOPA procedure. Both results are presented as mg of Nitrogen per liter (ppm) and the sum of the two is the total Yeast-Available Nitrogen as mg of Nitrogen per liter (YAN).

The nitrogen levels in grape juices intended for fermentation vary considerably. Some grapes have more than sufficient nitrogen for fermentation, but others are woefully inadequate. While 140 ppm available nitrogen has been mentioned as a minimum for healthy fermentation, current suggestions range from 200-350 ppm, depending on Brix.

MATERIALS

Reagents

- Sodium hydroxide/Boric acid Buffer
- N-acetyl-L-cysteine solution
- O-phthaldialdehyde in 95% ethanol (OPA)
- No-OPA solution (Reagent Alcohol)
- Isoleucine standard
- Deionized water

Glassware for diluting and filtering samples (beakers, pipettes, flasks)
Glass fiber filters & filter funnel
Repeatable pipettor(s), volumes 10 uL to 3 mL

Spectrophotometer at 335 nm, single beam or double beam
Methacrylic cuvettes, 10 mm path (polystyrene will do if necessary)
Holder for cuvettes
Calculation program (Excel or similar)

STANDARDS

The amino acid isoleucine (ILE) is used as a source of alpha-amino nitrogen. Alpha-amino groups react with O-phthaldialdehyde (OPA) to produce an absorbance increase at 335 nm. Known concentrations of the ILE standard are placed in a series of cuvettes. The ILE is allowed to react with OPA in a buffer solution to generate a standard curve.

Standard Tubes	**Blank**	**1**	**2**	**3**	**4**	**5**
10m*M* isoleucine (uL)	0	10	20	30	40	50
Distilled Water (uL)	50	40	30	20	10	0
Reagent Buffer + OPA (uL)	3000	3000	3000	3000	3000	3000
A335nm for 10 minutes						
Corresponding Nitrogent Concentration (mg/L)	0	28	56	84	112	140

ABOUT THE PROCEDURE

In enzymatic tests, the absorbance is measured on the same cuvette before and after the reaction takes place, so the influences on absorbance by the reagents, and by the sample itself, are constant in both measurements. But in this assay two series of tubes are set up to correct for the influence on absorbance of the reagents and the juice.

-No-OPA

First, the juice and reagent's absorbance is measured, so it can be subtracted from the absorbance after the reaction takes place, leaving only the change in absorbance due to the reaction. The juice is combined with all the reagents except for the reaction chemical (OPA). The spectrophotometer is zeroed on a tube with the reagents, except for OPA, and without any juice.

-With-OPA

Then cuvettes are set up for the reaction. They contain all reagents, including OPA, plus the juice sample, and the reaction takes place within 10 minutes. The spectrophotometer is zeroed against a tube with all the reagents, including OPA, but no juice.

A set of 5 standards is also run at the same time, using isoleucine as a source of amino nitrogen to react with the OPA. These tubes are prepared like the sample tubes, with all the reagents, including OPA. Again, the spectrophotometer is zeroed on the tube containing all the reagents, including OPA, but no sample.

PROCEDURE

Sample Preparation

Samples should be refrigerated or frozen until testing. Nitrogen is depleted very quickly during yeast growth, so if the sample begins fermenting the results will be much lower in nitrogen than it was initially.

Dilute juice with distilled water 1:1 unless it is likely to be very low (under 100 ppm). Test wine undiluted. Must may need dilution if fermentation is just beginning. Filter cloudy samples through glass fiber paper.

Setting up the Cuvettes

The hardest part of the test is to get the right solutions in the right tubes.

The tubes are grouped as follows:

	# tubes	called	contains
NO OPA:	1 (or 2)	"NO-OPA Blank"	buffer/alcohol mixture
	1 per sample	"Sample Blank"	buffer/alcohol mixture, sample
WITH OPA:	1 (or 2)	"WITH-OPA Blank"	buffer/OPA mixture
	2 per sample	"Sample"	buffer/OPA mixture, sample
	5	"Standards"	buffer/OPA mixture, ILE standards

Set methyacrylate cuvettes up in a rack in the pattern shown in the diagram on page 7 or in a similar pattern.

Preparing the Reagents

1. Calculate approximately how much NaOH/Boric acid/NAC buffer will be needed. You will need 3 ml per cuvette. Two tubes for each reagent blank are needed if the spectrophotometer has a double light beam, only one if it is a single-beam instrument.

		WITH OPA	NO OPA
Reagent blanks	6 ml or 12 ml (single/double beam)	3 ml/6 ml	3 ml/6 ml
Samples & sample blanks (per sample)	9 ml	6 ml	3 ml
Standards	15 ml	15 ml	0

Also calculate how much Buffer WITH OPA is needed and how much NO-OPA Buffer is needed.

2. **NO-OPA BUFFER:** Mix equal volumes of NaOH/Boric acid buffer and NAC solution together. Add reagent alcohol (95%).

Volume	NaOH/Boric Acid	NAC	Alcohol
10 ml	4.5 ml	4.5 ml	1 ml
50 ml	22.5 ml	22.5 ml	5 ml
100 ml	45 ml	45 ml	10 ml
200 ml	90 ml	90 ml	20 ml
300 ml	135 ml	135 ml	30 ml
400 ml	180 ml	180 ml	40 ml
500 ml	225 ml	225 ml	50 ml

3. **WITH-OPA BUFFER:** Mix equal volumes of NaOH/Boric acid buffer and NAC solution together. Add 0.67% OPA solution.

Volume	NaOH/Boric Acid	NAC	OPA
10 ml	4.5 ml	4.5 ml	1 ml
50 ml	22.5 ml	22.5 ml	5 ml
100 ml	45 ml	45 ml	10 ml
200 ml	90 ml	90 ml	20 ml
300 ml	135 ml	135 ml	30 ml
400 ml	180 ml	180 ml	40 ml
500 ml	225 ml	225 ml	50 ml

These solutions are stable in the refrigerator for about 5 days.

Adding Reagents

1. Into the “NO-OPA” cuvettes, dispense 3 ml of NO-OPA Buffer. Leave 1 (or 2 if a double-beam spectrophotometer is used) as a NO-OPA Blank.
2. Into one NO-OPA cuvette per sample, dispense 50 uL of diluted, filtered sample. These are the “SAMPLE BLANK” tubes.
3. Into the “WITH-OPA” cuvettes, dispense 3 ml of WITH-OPA Buffer. Leave one (or two) as a WITH-OPA Blank.
4. Into two cuvettes per sample, dispense 50 uL of diluted, filtered sample. These are the “SAMPLE” tubes.
5. Into the “STANDARD” tubes, dispense the following amounts of ILE and WATER:

	1	2	3	4	5
ILE	10 uL	20 uL	30 uL	40 uL	50 uL
H2O	40 uL	30 uL	20 uL	10 uL	0

6. Cover each cuvette with parafilm and mix by inverting several times. Allow to stand 10 minutes, no longer.
7. Put the NO-OPA BLANK tube(s) into the spectrophotometer at 335 nm and zero the instrument on this tube(s).

If the spectrophotometer has a single beam (one cuvette holder), put the blank tube in the holder, zero the instrument, and remove the tube. For a double-beam instrument (2 cuvette holders), put both blank tubes in the holders, auto-zero the instrument, and remove only the front blank tube, leaving the remaining tube in the instrument throughout the next readings.

8. In turn, read the absorbance of each SAMPLE BLANK.
9. Put the WITH-OPA BLANK tube(s) into the spectrophotometer and zero the instrument on this tube(s). (See step 7)
10. Read the absorbance of the SAMPLE tubes and STANDARD tubes.

Calculations

1. Use the absorbances of the standards to plot a curve. Use a basic plotter program, graph paper, or a spreadsheet program. Calculate slope and intercept.
2. Subtract the absorbance of the SAMPLE BLANK from the absorbance of the corresponding SAMPLE tubes.
3. Calculate the amount of alpha-amino nitrogen of the samples from the curve or spreadsheet. Multiply by the dilution factor (usually 2).

Nitrogen ppm = Absorbance(sample) x slope of curve + intercept x dilution factor

Report as ppm (mg/l) Nitrogen. Average the results from both cuvettes. If the replicates differ by 10-15%, retest if desired. If they differ by more than 15%, retesting is recommended.

Setting up Cuvettes			Adding Reagents	
NO-OPA		No-OPA blank	3 ml No-OPA buffer	
		sample #1	3 ml No-OPA buffer	50 µl sample #1 (filtered)
		sample #2	3 ml No-OPA buffer	50 µl sample #2 (filtered)
With-OPA		With-OPA blank	3 ml With-OPA buffer	
		sample #1	3 ml With-OPA buffer	50 µl sample #1 (filtered)
		sample #1 dup	3 ml With-OPA buffer	50 µl sample #1 (filtered)
		sample #2	3 ml With-OPA buffer	50 µl sample #2 (filtered)
		sample #2 dup	3 ml With-OPA buffer	50 µl sample #2 (filtered)
		standard #1 (28 ppm)	3 ml With-OPA buffer	10 µl ILE std & 40 µl water
		standard #2 (56 ppm)	3 ml With-OPA buffer	20 µl ILE std & 30 µl water
		standard #3 (84 ppm)	3 ml With-OPA buffer	30 µl ILE std & 20 µl water
		standard #4 (112 ppm)	3 ml With-OPA buffer	40 µl ILE std & 10 µl water
		standard #5 (140 ppm)	3 ml With-OPA buffer	50 µl ILE std & 00 µl water

81 M Street
Fresno, CA 93721
Tel: 559-485-2692
Fax: 559-485-4254

640-D Airpark Rd.
Napa, CA 94558
Tel: 707-224-7903
Fax: 707-255-2019

1165 Globe Ave.
Mountainside, NJ 07092
Tel: 908-301-1811
Fax: 908-301-1812

1401 Ware St.
Waupaca, WI 54981
Tel: 715-258-5525
Fax: 715-258-8488