Megazyme

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PRIMARY AMINO NITROGEN (PAN)

ASSAY PROCEDURE

K-PANOPA 11/20

(*100 Manual Assays per Kit) or
(1100 Auto-Analyser Assays per Kit) or
(1000 Microplate Assays per Kit)

**The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

The addition of nutrient supplements to grape juice prior to/during fermentation can now be managed accurately in terms of Yeast Available Nitrogen (YAN). YAN is especially important to determine accurately, as too little available nitrogen can result in sluggish or "stuck" fermentation and the generation of hydrogen sulphide (H_2S) gas, while too much nitrogen, such as in the form of added diammonium phosphate (DAP), can lead to the formation of the carcinogenic compound ethyl carbamate, especially where starting levels of L-arginine in the juice are high.

Total YAN (YAN_T) is comprised of three components:
(a) free ammonium ions,[1]
(b) primary amino nitrogen (PAN, from free amino acids),[2] and
(c) the contribution from the side chain of L-arginine (after hydrolysis by yeast arginase that creates ornithine and urea).[1]

All three components must therefore be measured accurately before any informed decision can be made regarding the addition of extra YAN in the form of DAP or yeast extract (permitted nutrient supplements). The YAN from ammonia and L-arginine (YAN_{AG}) (**a** plus **c**) can be conveniently and rapidly determined using the Megazyme L-Arginine/Urea/Ammonia kit (**K-LARGE**); the PAN component of YAN (**b**) is measured using the Megazyme Primary Amino Nitrogen kit (**K-PANOPA**). Total YAN is determined using both the K-PANOPA and K-LARGE kits, but because both measure the primary amino group of L-arginine, this has to be allowed for in the calculations (see page 6 of this booklet).

The PAN assay procedure (**K-PANOPA**) described in this booklet is a more accurate alternative to formol titration that also measures proline, an abundant amino acid found in grape juice that does not contribute to YAN in anaerobic fermentations.

PRINCIPLE:

The amino nitrogen groups of free amino acids in the sample react with *N*-acetyl-L-cysteine and *o*-phthaldialdehyde to form isoindole derivatives (1).[2]

(1) Amino nitrogen + *N*-acetyl-L-cysteine + *o*-phthaldialdehyde

isoindole derivative

The amount of isoindole derivative formed in this reaction is stoichiometric with the amount of free amino nitrogen. It is the isoindole derivative that is measured by the increase in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for amino acids containing primary amino groups, i.e. L-proline does not react as it contains a secondary amino group.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.65 mg/L of sample solution with a sample volume of 0.05 mL. The detection limit is 2.59 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 0.05 mL.

The assay is linear over the range of 0.2 to 10 µg of amino nitrogen per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.05 mL, this corresponds to an amino nitrogen concentration of approx. 0.65 to 1.30 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

Ammonium ions within the sample (e.g. a wine or must sample) result in an initial small increase in absorbance. This contribution to the total absorbance disappears however within the first 10 min of the reaction, and thus the assays must be allowed to react for 15 min before the absorbance readings are taken.
Samples of pure ammonium ions (e.g. DAP or ammonium sulphate) will be detected with a recovery rate of ~ 8%.

If the conversion of free amino nitrogen groups has been completed within the time specified in the assay (approx. 15 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding free amino nitrogen groups in the form of isoleucine (7 µg of nitrogen in 0.05 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding isoleucine to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 100 assays in manual format (or 1100 assays in auto-analyser format or 1000 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Tablets (100) containing *N*-acetyl-L-cysteine (NAC).
Stable for > 2 years at 4°C or below -10°C.

Bottle 2: Ortho-phthaldialdehyde (OPA) in 12 mL of ethanol (96% v/v).
Stable for > 2 years when stored in the dark at 4°C.

Bottle 3: Isoleucine standard solution (5 mL, 140 mg of nitrogen/L).
Stable for > 2 years; store sealed at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dissolve one tablet from bottle 1 in 3 mL of distilled water for each assay planned and allow to dissolve over 2-3 min. Aid dissolution by shaking, stirring or crushing with a metal spatula. **This is Solution 1.** Prepare immediately prior to use.

NOTE: Warm the tablet bottle to room temperature and remove any moisture from the outside of the container using a tissue before opening and removal of tablets. Opening the tablet bottle while it is cold will lead to absorption of moisture by the tablets which in turn will reduce the stability of the tablet components.

2. Use the contents of bottle 2 as supplied. This solution is stable for > 2 years when stored in the dark at 4°C.

3. Use the contents of bottle 3 as supplied.
Stable for > 2 years; store sealed at 4°C.

NOTE: The isoleucine standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of primary amino nitrogen is determined directly from the equation given on page 6.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® [to dispense 0.1 mL aliquots of OPA (solution 2)].
   - with 25 mL Combitip® [to dispense 3 mL aliquots of distilled water and NAC/buffer (solution 1)].
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 3.15 mL
Sample solution: 0.20-10.0 µg of nitrogen per cuvette (in 0.05 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
solution 1 (NAC/buffer) distilled water sample	3.00 mL 0.05 mL -	3.00 mL - 0.05 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:		
solution 2 (OPA)	0.10 mL	0.10 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 15 min).		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

NOTE: OPA is sensitive to light. Consequently, reactions must be performed in the dark (e.g. in the spectrophotometer cuvette compartment with the photometer lid closed).

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA_{PAN}.

The value of ΔA_{PAN} should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of PAN (primary amino nitrogen) can be calculated as follows:

$$c = \frac{V \times MW \times 1000}{\varepsilon \times d \times v} \times \Delta A_{PAN} \quad \text{[mg of N/L]}$$

where:

V = final volume [mL]
MW = molecular weight of nitrogen [g/mol]
1000 = conversion from g to mg of N/L
ε = extinction coefficient of isoindole derivative at 340 nm
= 6803 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for PAN:

$$c = \frac{3.15 \times 14.01 \times 1000}{6803 \times 1.0 \times 0.05} \times \Delta A_{PAN} \quad \text{[mg of N/L]}$$

$$= 129.74 \times \Delta A_{PAN} \quad \text{[mg of N/L]}$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

Yeast Available Nitrogen (YAN) as mg of N/L:

YAN is best estimated as the sum of its three principal components, i.e. the contribution from primary amino nitrogen (PAN), ammonia and the side-chain of L-arginine.[3] However, during fermentation urea may be excreted by the yeast after release from L-arginine. Thus if YAN is determined after fermentation begins, urea should also be included in the calculation. Various factors such as yeast strain used, the level of L-arginine, and the levels of other nitrogen sources,

namely free ammonium ions and amino acids, will determine the amount of urea excreted, and any subsequent re-utilisation. For further information regarding the interplay of these factors, and the significance of ethyl carbamate formation.[4] As the levels of these compounds vary widely, each component must be determined for an accurate YAN value to be obtained. The YAN from ammonia, urea and L-arginine (YAN_{AUG}) can be conveniently and rapidly determined using the Megazyme L-Arginine/Urea/Ammonia kit (**K-LARGE**) as follows:

$$YAN_{AUG} = 1000 \times \left[\frac{AM\ (g/L) \times 14.01}{17.03} + \frac{UR\ (g/L) \times 28.02}{60.06} + \frac{AR\ (g/L) \times 42.03}{174.21} \right]$$

[mg of N/L]

This calculation is based on one available nitrogen atom from each ammonium ion, two from urea and three available nitrogen atoms from each L-arginine molecule.[1,2] However, as L-arginine concentration varies widely in grape juice, and only represents approximately one third of all amino acids found at the most, accurate Total YAN values (YAN_T) are only obtained when the contribution from other primary amino nitrogen (PAN) groups is also determined. This can easily be achieved using this Primary Amino Nitrogen kit (**K-PANOPA**). In this case, it is important not to count the primary amino group of L-arginine again, and thus the equation becomes:

$$YAN_T = 1000 \times \left[\frac{AM\ (g/L) \times 14.01}{17.03} + \frac{UR\ (g/L) \times 28.02}{60.06} + \frac{AR\ (g/L) \times 28.02}{174.21} \right] + PAN$$

[mg of N/L]

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for primary amino nitrogen can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of primary amino nitrogen **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (tablets) distilled water	10 tablets 30 mL
Total volume	30 mL

Preparation of R2:

Component	Volume
bottle 2 (OPA) distilled water	1 mL 2 mL
Total volume	3 mL

EXAMPLE METHOD:

R1:	0.250 mL
Sample:	~ 0.005 mL
R2:	0.025 mL
Reaction time:	~ 15 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	~ 1 day
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.2 g/L of nitrogen using 0.005 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for primary amino nitrogen can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of primary amino nitrogen **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.315 mL
Linearity: 0.2-1.0 µg of nitrogen per well (in 0.005 mL sample volume)

Pipette into wells	Blank	Sample	Standard
solution 1 (NAC/buffer)	0.300 mL	0.300 mL	0.300 mL
distilled water	0.005 mL	-	-
sample solution	-	0.005 mL	-
standard solution	-	-	0.005 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
solution 2 (OPA)	0.010 mL	0.010 mL	0.010 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 15 min).			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

Sample dilution.

The amount of PAN present in the cuvette (i.e. in the 0.05 mL of sample being analysed), should range between 0.2 and 10 µg. The sample solution must therefore be diluted sufficiently to yield a primary amino nitrogen concentration between 4 and 200 mg N/L.

Dilution Table

Estimated concentration of PAN (mg N/L)	Dilution with water	Dilution factor (F)
< 200	No dilution required	1
200-2000	1 + 9	10

If the value of ΔA_{PAN} is too low (e.g. < 0.100), weigh out more sample or dilute less strongly.

SAMPLE PREPARATION EXAMPLE:

Determination of primary amino nitrogen in grape juice/ must and wine.

Generally, the concentration of primary amino nitrogen in white and red grape juice/must and wine can be determined without any sample treatment (except filtration and dilution according to the dilution table, if necessary). *Typically, no dilution is required and a sample volume of 50 µL is satisfactory.*

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