Megazyme

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L-ARGININE/UREA/ AMMONIA (*Rapid*)

(YEAST AVAILABLE NITROGEN; YAN)

ASSAY PROCEDURE

K-LARGE 08/18

(*50 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 quantify 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 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]

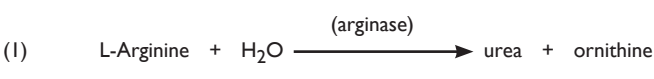
(c) the contribution from the side chain of L-arginine (after hydrolysis by yeast arginase that creates ornithine and urea).[1]

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 pages 7-8 of this booklet).

The rapid assay procedure (**K-LARGE**) described in this booklet enables the determination of both ammonium ions and L-arginine in a simple sequential format. Urea can also be quantified by this procedure and the levels of this compound should be minimised to prevent significant production of ethyl carbamate.

PRINCIPLE:

L-Arginine is hydrolysed to urea and ornithine by the enzyme arginase (1). The urea liberated is hydrolysed to ammonia (NH_3) and carbon dioxide (CO_2) by the enzyme urease (2).



In the presence of glutamate dehydrogenase (GlDH) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), ammonia (as ammonium ions; NH_4^+) reacts with 2-oxoglutarate to form L-glutamic acid and $NADP^+$ (3).

(3) 2-Oxoglutarate + NADPH + NH_4^+ $\xrightarrow{\text{(GlDH)}}$ L-glutamate + $NADP^+$ + H_2O

The amount of $NADP^+$ formed is stoichoimetric with the amount of ammonia. For each mole of L-arginine or urea hydrolysed, two moles of NADPH are consumed. NADPH consumption is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for L-arginine, urea and ammonia. In the analysis of reagent grade L-arginine, urea and ammonia, results of approx. 100% can be expected.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.0929 mg of L-arginine (or 0.0177 mg of ammonia/0.0318 mg of urea)/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.368 mg of L-arginine (or 0.0708 mg of ammonia/0.126 mg of urea)/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 1.0 to 35 mg of L-arginine, 0.2 to 7.0 µg of ammonia and 0.3 to 14 µg of urea 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 2.0 mL, this corresponds to an L-arginine concentration of approx. 0.0929 to 0.186 mg/L (or 0.0177 to 0.0354 mg of ammonia/0.0315 to 0.0636 mg of urea/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:

Unlike other kits for L-arginine, urea and ammonia determination, this product employs a novel glutamate dehydrogenase that is not significantly inhibited by the variable levels of interfering compounds, such as tannins, found in grape juice, wines and other materials. Thus the reaction time is, in general, much faster and more consistent when comparing samples of grapes from different batches or seasons, than is found with other analytical products.

However interfering substances, if present within the sample, 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 L-arginine, urea or ammonia to the sample in the initial extraction steps.

If the conversion of L-arginine, urea and ammonia has been completed within the times specified in the "PROCEDURE" on page 5, it can generally be concluded that no interference has occurred. However, this can be further checked by adding L-arginine (10 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease in the absorbance should be observed.

In alkaline buffer solution, protein fragments may slowly release ammonia which can lead to a slow creep reaction. If necessary, this creep reaction can be accounted for by extrapolation of absorbance values back to the time of addition of the enzyme.

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 50 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer (18 mL, pH 8.0) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2:	NADPH. Lyophilised powder. Stable for > 5 years below -10°C.
Bottle 3:	Glutamate dehydrogenase suspension (1.1 mL). Stable for > 2 years at 4°C.
Bottle 4:	Urease solution (2.7 mL). Stable for > 2 years below -10°C.
Bottle 5:	Arginase suspension (1.1 mL). Stable for > 2 years at 4°C.
Bottle 6:	Ammonia (0.04 mg/mL) standard solution. Stable for > 2 years at 4°C.

Bottle 7: L-Arginine powder (2 g) standard.
Stable for > 2 years stored dry at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 12 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3, 5 & 6. Use the contents of bottles 3, 5 and 6 as supplied. **Swirl bottles 3 & 5 to mix contents before use.**
Stable for > 2 years at 4°C.

4. Use the contents of bottle 4 as supplied. Store the bottle in an upright position.
Stable for > 2 years below -10°C.

7. Accurately weigh approx. 100 mg of L-arginine into a 1 L volumetric flask, fill to the mark with distilled water and mix thoroughly.
Stable for 3 months below -10°C.

NOTE: The L-arginine and ammonia standard solutions are 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 concentrations of L-arginine, urea and ammonia are determined directly from the extinction coefficient of NADPH (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® (20 µL and 100 µL).
4. Analytical balance.
5. Spectrophotometer set at 340 nm.
6. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
7. Stop clock.
8. Whatman No. 1 (9 cm) filter papers.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.62 mL (ammonia)
2.67 mL (urea)
2.69 mL (L-arginine)
Sample solution: 0.2-7.0 µg of ammonia per cuvette
0.3-14 µg of urea per cuvette
1.0-35 µg of L-arginine per cuvette
(in 0.1 to 2.0 mL sample volume)

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

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.30 mL	0.30 mL
solution 2 (NADPH)	0.20 mL	0.20 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions immediately by addition of:		
suspension 3 (GlDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) after approx. 5 min. Then add**:		
solution 4 (Urease)	0.05 mL	0.05 mL
Mix*, read the absorbances of the solutions (A_3) after approx. 6 min. Then add**:		
suspension 5 (Arginase)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_4) at the end of the reaction (approx. 7 min).		

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

** if a urea determination is unnecessary, e.g. when analysing fresh grape juice, the urease and arginase can be added together to save time.

Note: in this case, 0.07 mL of the enzyme mixture is used, and the value A_2-A_4 is used to calculate the amount of L-arginine, and not A_3-A_4 (see calculations section on next page).

CALCULATION:

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{ammonia}$.

Determine the absorbance difference (A_2-A_3) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA_{urea}.

Determine the absorbance difference (A_3-A_4) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{L\text{-}arginine}$.

For samples containing just ammonia and L-arginine, e.g. fresh grape juice (and where the urease and arginase are added together), determine the absorbance difference (A_2-A_4) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{L\text{-}arginine}$.

The values of $\Delta A_{ammonia}$, ΔA_{urea} and $\Delta A_{L\text{-}arginine}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of ammonia, urea and L-arginine can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of ammonia, urea or L-arginine [g/mol]
ε = extinction coefficient of NADPH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]
2 = 2 molecules of NADPH are consumed for each molecule of L-arginine or urea hydrolsed.

It follows for ammonia (AM):

$$c = \frac{2.62 \times 17.03}{6300 \times 1.0 \times 0.1} \times \Delta A_{ammonia} \quad [g/L]$$

$$= 0.07082 \times \Delta A_{ammonia} \quad [g/L]$$

for urea (UR):

$$c = \frac{2.67 \times 60.06}{6300 \times 1.0 \times 0.1 \times 2} \times \Delta A_{urea} \quad [g/L]$$

$$= 0.1273 \times \Delta A_{urea} \quad [g/L]$$

for L-arginine (AR):

$$c = \frac{2.69 \times 174.21}{6300 \times 1.0 \times 0.1 \times 2} \times \Delta A_{L\text{-arginine}} \quad [g/L]$$

$$= 0.3719 \times \Delta A_{L\text{-arginine}} \quad [g/L]$$

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, see reference 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 this L-Arginine/Urea/Ammonia kit (**K-LARGE**) as follows:

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

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 Megazyme's Primary Amino Nitrogen kit (**K-PANOPA**). In this case, it is important not to

count the primary amino group of L-arginine again; thus the equation becomes:

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

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

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of ammonia

$$= \frac{c_{ammonia}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

Content of urea

$$= \frac{c_{urea}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

Content of L-arginine

$$= \frac{c_{L\text{-}arginine}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

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).

SAMPLE PREPARATION:

1. Sample dilution.

The amount of analyte present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.2 and 7.0 µg (ammonia), 0.3 and 14 µg (urea) and 1.0 and 35 µg (L-arginine). The sample solution must therefore be diluted sufficiently to yield concentrations of ammonia between 0.01 and 0.08 g/L, urea between 0.02 and 0.14 g/L, and L-arginine between 0.05 and 0.40 g/L.

Dilution table

Estimated concentration of L-arginine (g/L)	Dilution with water	Dilution factor (F)
< 0.4	No dilution required	1
0.40-4.0	1 + 9	10
4.0-40	1 + 99	100

If the value of ΔA due to the analyte under investigation is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.0 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.1 mL and using the new sample volume in the equation.

2. Sample clarification.

Carrez reagents cannot be used for deproteinisation as their use results in significantly reduced recoveries. Perchloric or trichloroacetic acid are used as alternatives.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 8.0 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 8.0 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no GIDH, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP) per 10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of ammonia and L-arginine in grape juice.
Generally, the concentration of ammonia and L-arginine in white and

red grape juice 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 25-100 µL is satisfactory.*

(b) Determination of ammonia, urea and L-arginine in fermenting must and wine.

Generally, the concentration of ammonia, urea and L-arginine in white and red fermenting 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 25-100 µL is satisfactory.*

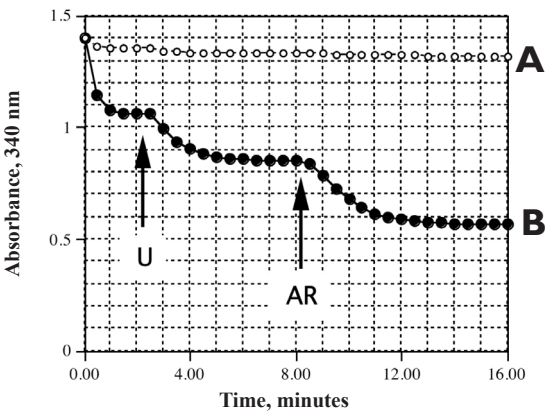


Figure 1. Decrease in absorbance at 340 nm on incubation of an ammonia/urea/L-arginine standard mixture with glutamate dehydrogenase in the presence of NADPH. **A**. blank; **B**. 2 µg of ammonia, 2.4 µg of urea and 10 µg of L-arginine. Urease (**U**) and arginase (**AR**) were added at the points shown by the arrows.

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4. Butzke, C. E. & Bisson, L. F. (1997). USFDA Center for Food Safety and Applied Nutrition, "*Ethyl Carbamate Preventative Action Manual*".

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