Megazyme

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AMMONIA
(*Rapid*)

ASSAY PROCEDURE

K-AMIAR 04/18

(For the rapid assay of ammonia in all samples, including grape juice and wine)

(*96 Manual Assays per Kit) or
(960 Auto-Analyser Assays per Kit) or
(960 Microplate Assays per Kit)

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

INTRODUCTION:

Ammonia is a widely occurring natural compound, often produced as a consequence of microbial protein catabolism, and thus serves as a quality indicator of fruit juice, milk, cheese, meat, seafood and bakery products. Unlike some other kits, this kit benefits from the use of a glutamate dehydrogenase that is not inhibited by tannins found in, for example, grape juice and wine. **K-AMIAR** can be used to determine ammonia manually (see page 4, "A"), in auto-analyser format (see page 6, "B"), or in microplate format (see page 7, "C"). In the wine industry, ammonia determination is important in the calculation of yeast available nitrogen (YAN). YAN comprises three highly variable components, free ammonium ions, primary amino nitrogen (from free amino acids), and the contribution from the side chain of L-arginine.[1] Thus, for the most accurate determination of YAN, all three components should be quantified, and this is possible using Megazyme's L-Arginine/Urea/Ammonia kit (**K-LARGE**) and NOPA kit (**K-PANOPA**).

PRINCIPLE:

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 $NADP^+$ (1).

(1) 2-Oxoglutarate + NADPH + NH_4^+ $\xrightarrow{\text{(GlDH)}}$ L-glutamic acid + $NADP^+$ + H_2O

The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia. It is NADPH consumption which is measured by the decrease in absorbance at 340 nm.[2]

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for ammonia. In the analysis of reagent grade ammonium sulphate, results of approx. 100% can be expected.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.018 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.071 mg/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 0.2 to 7 µg of ammonia per assay (Figure 2). In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to an ammonia concentration of approx. 0.018 to 0.035 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:

If the conversion of ammonia has been completed within the time specified in the assay (approx. 3 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding ammonia (approx. 4 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant decrease 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 ammonia to the sample in the initial extraction steps.

In alkaline buffer solution, protein fragments may slowly release ammonia which can lead to a slow creep reaction. This is not a problem with this kit, because the reaction is completed so quickly.

Tannins in fruit juice can lead to significant inhibition of GlDH from beef liver, the enzyme employed in Ammonia and Urea/Ammonia kits supplied by others. However, the enzyme used in this kit does not suffer from this limitation (Figure 3).

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 96 assays in manual format (or 960 assays in auto-analyser format or 960 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (36 mL, pH 8.0) plus 2-oxoglutarate and sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.

Bottle 2: (x 2) NADPH. Stable for > 5 years below -10°C.

Bottle 3: Glutamate dehydrogenase suspension (2.2 mL). Stable for > 2 years at 4°C.

Bottle 4: Ammonia standard solution (5 mL, 0.04 mg/mL) in 0.02% (w/v) sodium azide.
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS:

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

2. Dissolve the contents of one 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 & 4. Use the contents of bottles 3 and 4 as supplied. Store the bottles in an upright position. **Swirl bottle 3 to mix contents before use.** Stable for > 2 years at 4°C.

NOTE: The ammonia 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 ammonia is determined directly from the extinction coefficient of NADPH (page 5).

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 12.5 mL Combitip® [to dispense 0.5 mL aliquots of NADPH buffer (solution 2)].
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
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: 2.62 mL
Sample solution: 0.2-7.0 µg of ammonia per cuvette (in 0.1-2.0 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 by addition of:		
suspension 3 (GlDH)	0.02 mL	0.02 mL
Mix* and read the absorbance of the solutions (A_2) at the end of the reaction (~ 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances either remain the same, or increase constantly over 1 min**.		

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

** if this "creep" rate is greater for the sample than for the blank, extrapolate the absorbances (sample and blank) back to the time of addition of suspension 3.

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 $\Delta A_{ammonia}$.

The value of $\Delta A_{ammonia}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of ammonia can be calculated as follows:

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

where:

V = final volume [mL]
MW = molecular weight of ammonia [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]

It follows for ammonia:

$$c = \frac{2.62 \times 17.03}{6300 \times 1.0 \times 0.10} \times \Delta A_{ammonia} \quad [g/L]$$

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

If the sample has been 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

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

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).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for ammonia 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 ammonia **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
distilled water solution 1 (buffer) bottle 2 (NADPH)	60 mL 12 mL 8 mL (after adding 12 mL of H_2O to bottle 2)
Total volume	80 mL

Preparation of R2:

Component	Volume
distilled water solution 1 (buffer) suspension 3 (GIDH)	6.8 mL 0.5 mL 0.7 mL
Total volume	8.0 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 5 min at 25°C or 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 7 days when refrigerated
Calculation:	endpoint
Reaction direction:	decrease
Linearity:	up to 62 mg/L of ammonia using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for ammonia 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 ammonia **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: 0.262 mL
Linearity: 0.1-0.7 µg of ammonia per well (in 0.01-0.2 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.030 mL	0.030 mL	0.030 mL
solution 2 (NADPH)	0.020 mL	0.020 mL	0.020 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
suspension 3 (GlDH)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 1 min intervals until the absorbances increase constantly over 1 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).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 3.

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.

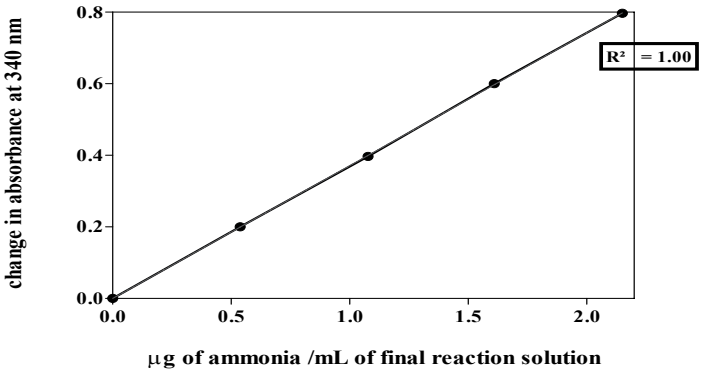

Figure 1. Calibration curve demonstrating the linearity of **K-AMIAR**. The reactions used to generate this calibration curve were performed at 37°C for 5 min, using a 10 mm path-length cuvette.

SAMPLE PREPARATION (Manual Format, A):

1. Sample dilution.

The amount of ammonia present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.2 and 7 µg. The sample solution must therefore be diluted sufficiently to yield an ammonia concentration between 0.01 and 0.07 g/L.

Dilution Table

Estimated concentration of ammonia (g/L)	Dilution with water	Dilution factor (F)
< 0.07	No dilution required	1
0.07-0.7	1 + 9	10
0.7-7.0	1 + 99	100

If the value of $\Delta A_{ammonia}$ 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 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 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 (see specific examples).

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 may need to be increased to approx. 8.0 using 2 M NaOH.

(c) Carbon dioxide: samples containing significant quantities of carbon dioxide 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: if required, a sample blank, i.e. sample with no GlDH, should be performed 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)/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.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask at 60°C. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate, and use the clear supernatant (which may be slightly opalescent) for assay.

(h) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge at 1,500 *g* for 10 min and neutralise the supernatant with 1 M KOH. Alternatively, use trichloroacetic acid as described in sample preparation example (c) below.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of ammonia in grape juice/must and wine.
Generally, the concentration of ammonia 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). If volumes greater than 25 µL of red wine are to be analysed, it may be necessary to remove some of the colour by the addition of 0.2 g of PVPP/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper. *Typically, no dilution is required and a sample volume of 25-50 µL is satisfactory.*

(b) Determination of ammonia in fruit juices (e.g. orange juice).

Adjust 25 mL of filtered sample to a pH of approx. 8.0 using 2 M NaOH. Quantitatively transfer the solution to a 50 mL volumetric flask and adjust to volume with distilled water. If the solution is highly coloured, it may be necessary to remove some of the colour by the addition of 0.2 g of PVPP/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper. *Typically, no dilution is required and a sample volume of 0.1 mL is satisfactory.*

(c) Determination of ammonia in milk.

In a glass test-tube, accurately mix 1 mL of milk with 3 mL of 0.3 M trichloroacetic acid. Incubate at room temperature for 5 min to ensure complete precipitation of protein and then centrifuge at room temperature for 3 min at 2,000 *g*. Decant supernatant and, using pH test strips, neutralise with 10 M KOH (the high concentration of KOH leading to an insignificant increase in volume). Filter, and use the clear supernatant directly for the assay. *Typically, no further dilution is required and sample volumes up to 2.0 mL will be required.*

(d) Determination of ammonia in baking products.

Accurately weigh approx. 10 g of representative material into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using an Ultra-turrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat. Filter, discarding the first 3-5 mL, and use the clear filtrate for the assay. *Typically, no further dilution is required and a sample volume of 0.5 mL is satisfactory.*

(e) Determination of ammonia in meat and meat products.

Accurately weigh approx. 5 g of representative material into a 100 mL Duran® bottle. Add 20 mL of 1 M perchloric acid and homogenise for 2 min using an Ultra-turrax® or Polytron® homogeniser (or equivalent). Quantitatively transfer to a 40 mL glass beaker and adjust the pH to approx. 8.0 using 2 M KOH. Quantitatively transfer to a 100 mL volumetric flask and adjust to the mark with distilled water (ensuring the fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Store on ice for 20 min to precipitate potassium perchlorate and allow separation of the fat. Filter, discarding the first 3-5 mL, and use the clear filtrate for the assay. *Typically, no further dilution is required and a sample volume of 0.5 mL is satisfactory.*

(f) Determination of ammonia in liquorice products.

Homogenise approx. 3 g of sample using a pestle and mortar and accurately weigh approx. 1 g of representative material into a 100 mL volumetric flask. Add 60 mL of distilled water and incubate at 70°C for 10 min, or until fully dissolved. Allow to equilibrate to room temperature and fill to the mark with distilled water. Filter and use the slightly coloured filtrate for the assay. *Typically, no further dilution is required and a sample volume of 0.5 mL is satisfactory.*

(g) Determination of ammonia in water (e.g. swimming pool water).

The ammonia concentration of water can generally be determined without any sample treatment. *Typically, no dilution is required and sample volumes up to 2.0 mL will be required.*

REFERENCES:

1. Martin, O., Brandriss, M. C., Schneider, G. & Bakalinsky, A. T. (2003). Improved anaerobic use of arginine by *Saccharomyces cerevisiae*. *App. Env. Microbiol.,* **69**, 1623-1628.

2. Bergmeyer, H. U. & Beutler, H. O. (1990). Ammonia. "*Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VIII**, pp. 454-461, VCH Publishers (UK) Ltd., Cambridge, UK.

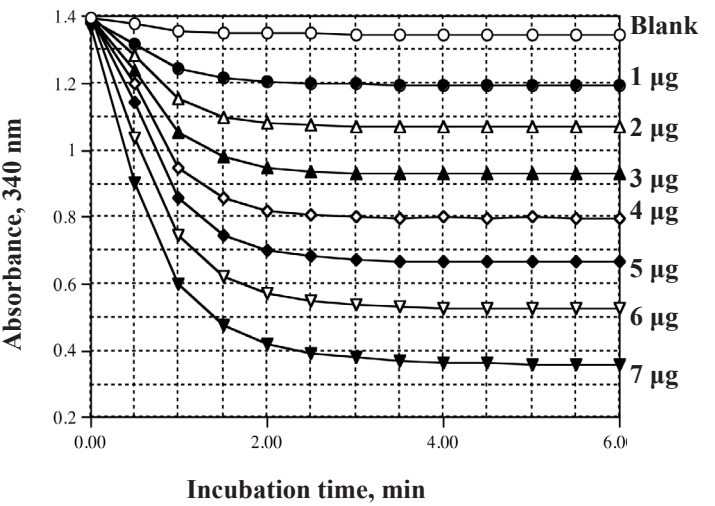

Figure 2. Decrease in absorbance at 340 nm on incubation of 1-7 µg of ammonia with glutamate dehydrogenase in the presence of NADPH.

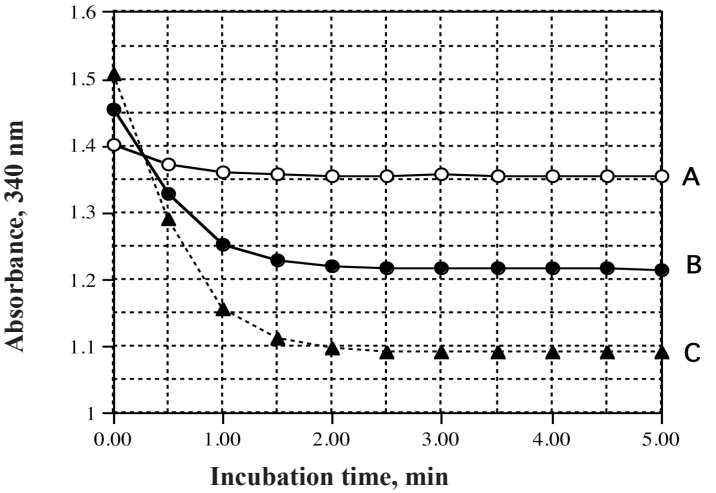

Figure 3. Decrease in absorbance at 340 nm on incubation of untreated red must preparation with glutamate dehydrogenase in the presence of NADPH. A. blank; B. 0.025 mL of red must sample; C. 0.05 mL of red must sample.

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