Megazyme

AMMONIA

ASSAY PROCEDURE

K-AMIA 08/04

(75 Assays per Kit)

Megazyme

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. Ammonia is also monitored in swimming pool water, waste water, and fermentation cultures, where its detection may indicate the presence of faeces, urine and microbes. Other applications include aqueous ammonia cleaning products, fertilisers, cosmetics, explosives, and as a refrigerant.

PRINCIPLE:

In the presence of glutamate dehydrogenase (GlDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia (as ammonium ions; NH_4^+) reacts with 2-oxoglutarate to form L-glutamic acid and NAD^+ (1).

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

The amount of NAD^+ formed is stoichiometric with the amount of ammonia. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

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 0.017 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.069 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 10 μg of ammonia 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 2.00 mL, this corresponds to an ammonia concentration of approx. 0.017 to 0.034 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. 10 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. If necessary, this creep reaction can be accounted for by extrapolation of absorbance value (A_2) back to the time of addition of solution 3 (GlDH).

Tannins in fruit juice can lead to some inhibition of GlDH, thus fruit juices should be routinely treated with polyvinylpolypyrrolidone (PVPP).

SAFETY:

The reagents used in the determination of ammonia are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 75 determinations are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	TEA buffer (20 mL, 1.0 M, pH 8.0) plus 2-oxoglutarate (100 mM) and sodium azide (0.02 % w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2: (x2)	NADH (18 mg) plus stabiliser. Stable for > 5 years at -20°C.
Bottle 3:	Glutamate dehydrogenase solution (4.0 mL, 200 U/mL). Stable for > 2 years at -20°C.
Bottle 4:	Ammonia standard solution (5 mL, 0.04 mg/mL). 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 bottle 2 in 8 mL distilled water. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. **Do not** dissolve the contents of the second bottle until required. When dissolved, the reagent is stable for > 12 months at -20°C.

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

4. Use the contents of bottle 4 as supplied.
Stable for > 2 years at room temperature.

> **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 NADH (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 and 200 μL).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of NADH solution and 0.2 mL aliquots of TEA buffer).
   - 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.

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.55 mL
Sample solution: 0.2-10 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) sample solution 1 (TEA buffer mix) solution 2 (NADH)	2.10 mL - 0.20 mL 0.20 mL	2.00 mL 0.10 mL 0.20 mL 0.20 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 5 min and start the reactions immediately by addition of:		
solution 3 (GlDH)	0.05 mL	0.05 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same or decrease constantly over 2 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 that of the blank extrapolate the absorbances (sample and blank) back to the time of the addition of solution 3 (GlDH).

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 NADH at 340 nm
= 6300 [$l \times mol^{-1} \times cm^{-1}$]
d = light path [cm]
v = sample volume [mL]

It follows for ammonia:

$$c = \frac{2.55 \times 17.03}{6300 \times 1.0 \times 0.10} \times \Delta A_{ammonia} \quad [g/L]$$

$$= 0.06893 \times \Delta A_{ammonia} \quad [g/L]$$

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

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

SAMPLE PREPARATION:

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 10 μg. The sample solution must therefore be diluted sufficiently to yield an ammonia concentration between 0.01 and 0.10 g/L.

Dilution Table

Estimated concentration of ammonia (g/L)	Dilution with water	Dilution factor (F)
< 0.10	No dilution required	1
0.10-1.0	1 + 9	10
1.0-10	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 an acidic sample is to be used undiluted (such as red wine or coloured 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 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: 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 1 g/100 mL of polyvinylpolypyrrolidone (PVPP). Stir for 2 min and then filter.

(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. Adjust to 20°C 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 (a) below.

SAMPLE PREPARATION EXAMPLES:

(a) 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.*

(b) 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 Ultraturrax® 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.*

(c) 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 Ultraturrax® 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.*

(d) Determination of ammonia in fruit juices.
Adjust 25 mL of fruit juice to pH 8.0 with 2 M KOH, quantitatively transfer to a 50 mL volumetric flask and fill to the mark with distilled water. Transfer the solution to a 100 mL beaker, add 1 g of PVPP and stir the suspension for 2 min on a magnetic stirrer. Filter an aliquot of the suspension and use the clear, slightly turbid solution for the assay. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

(e) 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 25°C 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.*

(f) 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.*

REFERENCE:

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

NOTES:

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