Megazyme

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UREA/AMMONIA (*Rapid*)

ASSAY PROCEDURE

K-URAMR 10/18

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

(*50 Assays of each per Kit)

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

INTRODUCTION:

Urea and ammonia are widely occurring natural compounds. As urea is the most abundant organic solute in urine, and ammonia is produced as a consequence of microbial protein catabolism, these analytes serve as reliable quality indicators for food products such as fruit juice, milk, cheese, meat and seafood. Ammonium carbonate is used as a leaven in baked goods such as quick breads, cookies and muffins. 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. In the wine industry, ammonia determination is important in the calculation of yeast available nitrogen (YAN). YAN is comprised of three highly variable components, free ammonium ions, primary amino nitrogen (from free amino acids) and the contribution from the sidechain of L-arginine.[1] 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**). Urea determination can be important in preventing the formation of the known carcinogen ethyl carbamate (EC) in finished wine.

PRINCIPLE:

Urea is hydrolysed to ammonia (NH_3) and carbon dioxide (CO_2) by the enzyme urease (1).

$$(1)\quad \text{Urea} + H_2O \xrightarrow{\text{(urease)}} 2NH_3 + CO_2$$

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 $NADP^+$ (2).

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

The amount of $NADP^+$ formed is stoichiometric with the amount of ammonia. For each mole of urea reacted, two moles of NADPH are consumed. NADPH consumption is measured by the decrease in absorbance at 340 nm.[2]

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for urea and ammonia. In the analysis of reagent grade urea and 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 of ammonia (or 0.031 mg urea)/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.071 mg of ammonia (or 0.1258 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 0.2 to 7 µg of ammonia (0.3 to 14 µg of urea) per assay (Figure 1, page 12). 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 (or 0.031 to 0.063 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:

If the conversion of urea and ammonia has been completed within the times specified in the assays, 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 (not supplied)] or urea (approx. 7 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 or urea 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 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 the **K-URAMR** kit does not suffer from this limitation (Figure 2, page 12).

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 (of each) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (18 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: 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: Urea control powder (~ 2 g). Stable for > 2 years at room temperature.

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 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. Use the contents of bottle 3 as supplied. Store the bottle in an upright position. **Swirl the bottle 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.
5. Accurately weigh approx. 70 mg of urea into a 1 L volumetric flask, fill to the mark with distilled water and mix thoroughly. Prepare fresh before use. This control solution is stable for ~ 3 months below -10°C.

NOTE: The urea 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 concentrations of ammonia and urea 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® (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 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 and GF/A (9 cm) glass fibre 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)
Sample solution: 0.2-7.0 µg of ammonia per cuvette
or 0.3-14.0 µg urea 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 immediately by addition of:		
suspension 3 (GlDH)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) after approx. 5 min. Then add**:		
solution 4 (Urease)	0.05 mL	0.05 mL
Mix* and read the absorbances of the solutions (A_3) 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 remain the same.		

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

This reaction sequence is shown in Figure 3 (page 13).

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}.

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

The concentration of ammonia and urea 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 the substance assayed [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:

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

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

For urea:

$$c = \frac{2.67 \times 60.06}{6300 \times 1.0 \times 0.10 \times 2} \times \Delta A_{urea} \quad [g/L]$$

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

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).

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

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

Content of urea

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of urea (ammonia) present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 0.3 and 14 µg (0.2 and 7 µg). The sample solution must therefore be diluted sufficiently to yield a urea (ammonia) concentration between 0.02 and 0.14 g/L (0.01 and 0.08 g/L).

Dilution Table

Estimated concentration of urea (ammonia) (g/L)	Dilution with water	Dilution factor (F)
< 0.14 (< 0.07)	No dilution required	1
0.14-1.4 (0.07-0.7)	1 + 9	10
1.4-14 (0.7-7.0)	1 + 99	100

If the value of $\Delta A_{ammonia}$ or ΔA_{urea} 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 point (h) Samples containing protein, on page 8].

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 significant quantities 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 GlDH, 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)/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 (b) below.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of urea and ammonia in grape juice/must and wine.

Generally, the concentration of urea and 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 with activated PVPP as described in "General considerations - (e) strongly

coloured samples". *However, typically, no dilution is required and a sample volume of 25-50 µL is satisfactory.*

(b) Determination of urea 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*. Use the clear supernatant directly for the assay. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

(c) Determination of urea and 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). Incubate at 4°C 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 urea and ammonia in water (e.g. swimming pool water).

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

(e) Determination of urea and 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, discard 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 urea and ammonia in fruit juices.

Adjust 25 mL of fruit juice to approx. 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.*

(g) Determination of urea and 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.*

(h) Determination of urea and ammonia in whole blood samples.

a. Solutions:

Concentrated Carrez I solution. Dissolve 30 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 200 mL of distilled water. Store at room temperature.

Concentrated Carrez II solution. Dissolve 60 g of zinc sulphate $\{ZnSO_4.7H_2O\}$ (Sigma cat. no. Z4750) in 200 mL of distilled water. Store at room temperature.

b. Procedure:

Heat 1 mL of whole blood sample at approx. 80°C for 20 min in a microfuge tube then centrifuge at 13,000 x *g* for 10 min and recover the supernatant. Add 20 µL Carrez Reagent II and mix thoroughly, then add 20 µL Carrez Reagent I and mix thoroughly. Centrifuge the sample again at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The final volume of the clarified supernatant will be approximately one quarter of the starting volume of the original sample. Therefore adjust the volume of the starting material as required to obtain sufficient volume of clarified sample for the test.

(i) Determination of urea and ammonia in biological tissue samples.

Accurately weigh approx. 5 g of representative biological tissue 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 (if present). Centrifuge an appropriate volume of the sample at 13,000 x *g* for 10 min and recover the clarified supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the clear filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay.

Note: The amount of starting material and volumes used can be adjusted accordingly depending on the amount of analyte present in the sample.

(j) Determination of urea and ammonia in biological fluid samples (e.g. urine and serum).

For some biological fluid samples it may be sufficient to test them directly without any sample preparation other than appropriate dilution in distilled water. If this is not adequate then deproteinisation with either perchloric acid or trichloracetic acid may be required.

Deproteinise biological samples by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Centrifuge an appropriate volume of the sample at 1,500 x *g* for 10 min and recover the supernatant for use in the assay, alternatively filter through Whatman No. 1 filter paper, discarding the first 3-5 mL, and use the filtrate for the assay. If required, dilute the sample appropriately in distilled water for the assay. Alternatively, use 50% (w/v) trichloroacetic acid instead of perchloric acid.

REFERENCES:

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2. Kerscher, L. & Ziegenhorn, J. (1990). Urea. "*Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VIII**, pp. 444-453, VCH Publishers (UK) Ltd., Cambridge, UK.

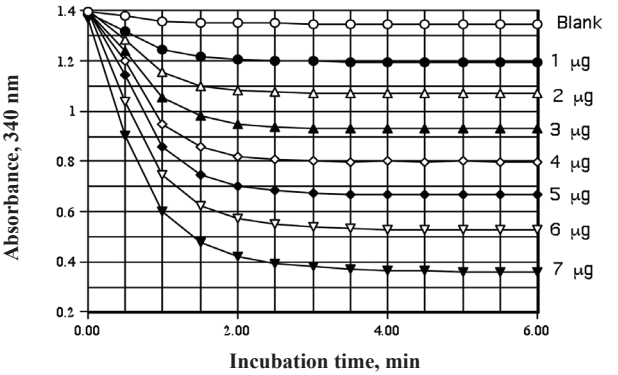

Figure 1. Decrease in absorbance at 340 nm on incubation of 1-7 µg of ammonia with glutamate dehydrogenase in the presence of NADPH.

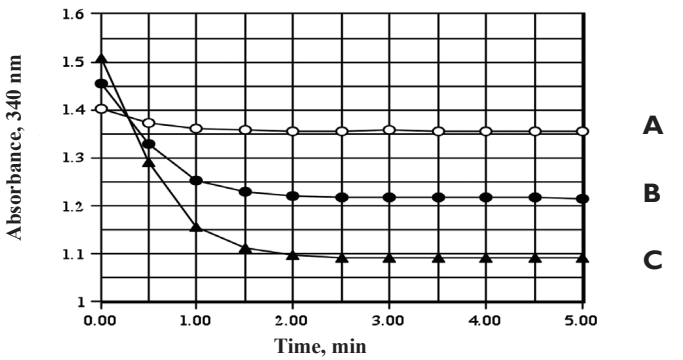

Figure 2. Decrease in absorbance at 340 nm on incubation of untreated red must preparation with glutamate dehydrogenase in the presence of NADPH. **A.** blank; **B.** 0.025 mL of red must sample; **C.** 0.05 mL of red must sample.

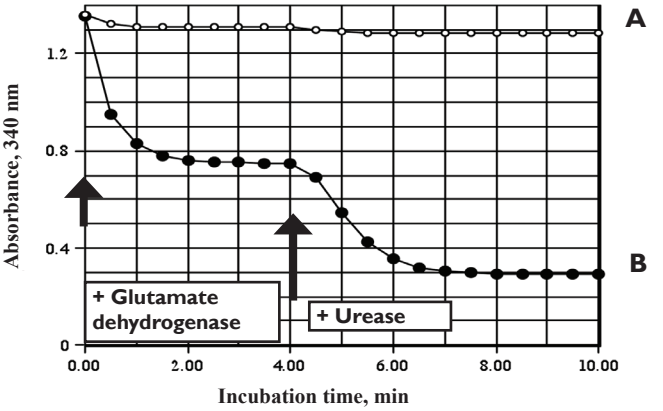

Figure 3. Decrease in absorbance at 340 nm on incubation of a urea/ammonia standard with glutamate dehydrogenase and urease in the presence of NADPH. **A.** blank; **B.** 4 µg of ammonia plus 7 µg of urea. Glutamate dehydrogenase and urease were added at the points shown by the arrows.

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