

# Megazyme

---

## UREA/AMMONIA

### ASSAY PROCEDURE

K-URAM 08/04

(40 Assays of each per Kit)

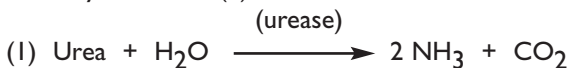


## INTRODUCTION:

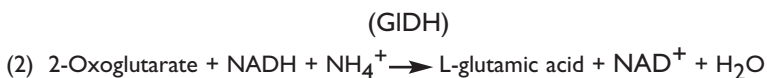
Urea and ammonia are widely occurring natural compounds. As urea is the most abundant organic solute in urine, and ammonia is produced as a consequence of microbial protein catabolism, these analytes serve as reliable quality indicators for food products such as fruit juice, milk, cheese, meat and seafood. Ammonium carbonate is used as a leaven in baked goods such as quick breads, cookies, and muffins. In the wine industry, urea levels are monitored, and reduced enzymatically if necessary, to prevent the formation of ethyl carbamate, a known carcinogen. Levels of urea and ammonia are also determined in swimming pool water, waste water, and fermentation cultures, where detection may indicate the presence of faeces, urine and microbial growth. Urea and ammonia are also found in cleaning products, fertilisers, cosmetics, explosives, and refrigerants.

## PRINCIPLE:

Urea is hydrolysed to ammonia ( $\text{NH}_3$ ) and carbon dioxide ( $\text{CO}_2$ ) by the enzyme urease (1).



In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia (as ammonium ions;  $\text{NH}_4^+$ ) reacts with 2-oxoglutarate to form L-glutamic acid and  $\text{NAD}^+$  (2).



The amount of  $\text{NAD}^+$  formed is stoichiometric with the amount of ammonia or with twice the amount of urea. 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 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 0.017 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 0.069 mg of ammonia or 0.124 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 10  $\mu\text{g}$  of ammonia (0.3 to 18  $\mu\text{g}$  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 2.00 mL, this corresponds to an ammonia concentration of approx. 0.017 to 0.034 mg/L (or 0.031-0.062 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  $\mu\text{g}$  in 0.1 mL (not supplied)] or urea (approx. 14  $\mu\text{g}$  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. If necessary, this creep reaction can be accounted for by extrapolation of absorbance value ( $A_2$ ) back to the time of addition of solution 4 (GIDH).

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

## SAFETY:

The reagents used in the determination of ammonia and urea 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 40 determinations of each analyte 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:** Urease solution (2.1 mL, 280 U/mL). Stable for > 2 years at -20°C.
- Bottle 4:** Glutamate dehydrogenase solution (4.1 mL, 200 U/mL). Stable for > 2 years at -20°C.
- Bottle 5:** Urea control powder (~ 2 g). 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 8.2 mL of 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 & 4. Use the contents of bottles 3 and 4 as supplied. Stable for > 2 years at 4°C.
5. Accurately weigh approx. 140 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 can be stored frozen in aliquots at -20°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 NADH (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  $\mu$ L and 200  $\mu$ L).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
  - with 5.0 mL Combitip® to dispense 0.2 mL aliquots of NADH and 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 and GF/A (9 cm) glass fibre filter papers.

## PROCEDURE:

<b>Wavelength:</b>	340 nm
<b>Cuvette:</b>	1 cm light path (glass or plastic)
<b>Temperature:</b>	~ 25°C
<b>Final volume:</b>	2.55 mL (ammonia); 2.60 mL (urea)
<b>Sample solution:</b>	0.2-10 µg of ammonia per cuvette or 0.3-18 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 urea	Urea sample	Blank ammonia	Ammonia sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL	2.10 mL	2.00 mL
sample	-	0.10 mL	-	0.10 mL
solution 1 (TEA buffer)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
solution 2 (NADH)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
solution 3 (urease)	0.05 mL	0.05 mL	-	-
Mix*, read the absorbances of the solutions ( $A_1$ ) after approx. 5 min and start the reactions immediately by addition of:				
solution 4 (GIDH)	0.05 mL	0.05 mL	0.05 mL	0.05 mL
Mix*, read the absorbances of the solutions ( $A_2$ ) at the end of the reaction (approx. 20 min). If the reaction has not stopped after 20 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 4 (GIDH).

## CALCULATION:

Determine the absorbance difference ( $A_1 - A_2$ ) for both blank and sample and calculate values of  $\Delta A_{\text{ammonia}}$  and  $\Delta A_{\text{urea + ammonia}}$ , as described below:

### Determination of ammonia:

$\Delta A_{\text{ammonia}} = (A_1 - A_2)_{\text{sample}} - (A_1 - A_2)_{\text{blank}}$  (from the ammonia sample).

### Determination of urea plus ammonia:

$\Delta A_{\text{urea + ammonia}} = (A_1 - A_2)_{\text{sample}} - (A_1 - A_2)_{\text{blank}}$  (from the urea sample).

### Determination of urea:

The difference between  $\Delta A_{\text{urea + ammonia}}$  (from the urea sample) and  $\Delta A_{\text{ammonia}}$  (from the ammonia sample) yields  $\Delta A_{\text{urea}}$ .

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

The concentration of ammonia or urea can be calculated as follows:

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

### where:

V = final volume [mL]

MW = molecular weight of the substance assayed [g/mol]

$\epsilon$  = extinction coefficient of NADH at 340 nm

$$= 6300 \text{ [l} \times \text{mol}^{-1} \times \text{cm}^{-1}\text{]}$$

d = light path [cm]

v = sample volume [mL]

### It follows for urea:

$$c = \frac{2.60 \times 60.06}{6300 \times 1.0 \times 0.10 \times 2} \times \Delta A_{\text{urea}} \quad [\text{g/L}]$$

$$= 0.1239 \times \Delta A_{\text{urea}} \quad [\text{g/L}]$$

### for ammonia:

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

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

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 urea

$$= \frac{C_{\text{urea}} [\text{g/L sample solution}]}{\text{weight}_{\text{sample}} [\text{g/L sample solution}]} \times 100 \quad [\text{g/100 g}]$$

### Content of ammonia

$$= \frac{C_{\text{ammonia}} [\text{g/L sample solution}]}{\text{weight}_{\text{sample}} [\text{g/L sample solution}]} \times 100 \quad [\text{g/100 g}]$$

## 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 18  $\mu\text{g}$  (0.2 and 10  $\mu\text{g}$ ). The sample solution must therefore be diluted sufficiently to yield a urea (ammonia) concentration between 0.02 and 0.18 g/L (0.01 and 0.10 g/L).

### Dilution Table

Estimated concentration of urea (ammonia) (g/L)	Dilution with water	Dilution factor (F)
< 0.18 (< 0.10)	No dilution required	1
0.18-1.8 (0.10-0.1)	1 + 9	10
1.8-18 (0.1-10.0)	1 + 99	100

If the value of  $\Delta A_{\text{urea}}$  or  $\Delta A_{\text{ammonia}}$  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 GIDH, 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 examples (a) below.

## SAMPLE PREPARATION EXAMPLES:

### (a) 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.

### **(b) 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 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). 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.*

### **(c) 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.*

### **(d) 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 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, 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.*

### **(e) Determination of urea and 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.*





**Megazyme International Ireland Ltd.,  
Bray Business Park, Bray,  
Co. Wicklow,  
IRELAND**

**Telephone: (353.1) 286 1220**

**Facsimile: (353.1) 286 1264**

**Internet: [www.megazyme.com](http://www.megazyme.com)**

**E-Mail: [info@megazyme.com](mailto:info@megazyme.com)**

---

---

**WITHOUT GUARANTEE**

The information contained in this booklet is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.

