Urea/Ammonia

UV-method

for the determination of urea and ammonia in foodstuffs and other materials and for the determination of nitrogen after Kjeldahl-digestion (see pt. 12.2)

Cat. No. 10 542 946 035

Test-Combination for approx. 25 determinations each

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For use in *in vitro* only

Store at 2-8° C

For recommendations for methods and standardized procedures see ref. (2)

Principle (Ref. 1)

Urea is hydrolyzed to ammonia and carbon dioxide in the presence of the enzyme urease (1).

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

In the presence of glutamate dehydrogenase (GIDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia reacts with 2-oxoglutarate to L-glutamate, whereby NADH is oxidized (2).

$$(2)\ \text{2-Oxoglutarate} + NADH + NH_4^+ \xrightarrow{\text{GIDH}} \text{L-glutamate} + NAD^+ + H_2O$$

The amount of NADH oxidized in the above reaction is stoichiometric to the amount of ammonia or with half the amount of urea, respectively. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

The Test-Combination contains

1. Bottle 1 with approx. 60 ml solution, consisting of: triethanolamine buffer, pH approx. 8.0; 2-oxoglutarate, approx. 220 mg
2. Bottle 2 with approx. 50 tablets; each tablet contains: NADH, approx. 0.4 mg
3. Bottle 3 with approx. 0.7 ml urease solution, approx. 80 U
4. Bottle 4 with approx. 1.2 ml glutamate dehydrogenase solution, approx. 1000 U

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve **one tablet** of bottle 2 with **one ml** solution of bottle 1 in a beaker or in a reagent tube for each assay (blank and samples) depending on the number of determinations. Use forceps for taking the tablets out of bottle 2. This results in reaction mixture 2*.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).
Bring solution 1 to 20-25°C before use.
Tablets 2 are stable at 2-8°C (see pack label).
Reaction mixture 2 is stable for 3 days at 2-8°C.
Bring reaction mixture 2 to 20-25°C before use.
The contents of bottle 3 and 4 are stable at 2-8°C (see pack label).

Procedure

Wave length[1]:	340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette[2]:	1.00 cm light path
Temperature:	20-25°C
Final volume:	3.040 ml
Read against air	(without a cuvette in the light path) or against water
Sample solution:	0.3-14 µg urea/assay[3] or 0.2-8 µg ammonia/assay[3] (in 0.100-2.000 ml sample volume)

Pipette into cuvettes	Blank urea	Urea sample	Blank ammonia	Ammonia sample
reaction mixture 2*	1.000 ml	1.000 ml	1.000 ml	1.000 ml
sample solution**	-	0.100 ml	-	0.100 ml
solution 3	0.020 ml	0.020 ml	-	-
redist. water	2.000 ml	1.900 ml	2.020 ml	1.920 ml
Mix***, and read absorbances of the solutions (A_1) after approx. 5 min at 20-25°C. Start reaction by addition of:				
solution 4	0.020 ml	0.020 ml	0.020 ml	0.020 ml
Mix***, wait for completion of the reaction (approx. 20 min) and read absorbances of the solutions (A_2). If the reaction has not stopped after 20 min, read absorbances in 2 min intervals until the absorbances decrease constantly over 2 min.				

If the absorbance A_2 decreases constantly, extrapolate the absorbance to the time of the addition of solution 4 (GIDH).

Determine the absorbance differences (A_1-A_2) for both, blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample.

$$\Delta A = (A_1\text{-}A_2)_{sample} - (A_1\text{-}A_2)_{blank}$$

This results in $\Delta A_{\text{urea + ammonia}}$ (from urea sample) and $\Delta A_{ammonia}$ (from ammonia sample).

The difference of these values results in ΔA_{urea}.

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt. 4).

If the absorbance differences of the samples (ΔA_{sample}) are higher than 1.000 (measured at 340 nm or Hg 334 nm respectively) or 0.500 (measured at 365 nm), the concentration of urea (or ammonia) in the sample solution is too high. The sample is to be diluted according to the dilution table in that case.

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\varepsilon \times d \times v \times 1000} \times \Delta A\ [g/l]$$

V = final volume [ml]

v = sample volume [ml]

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

d = light path [cm]

ε = extinction coefficient of NADH at:
340 nm = 6.3 [l × mmol^{-1} × cm^{-1}]
Hg 365 nm = 3.4 [l × mmol^{-1} × cm^{-1}]
Hg 334 nm = 6.18 [l × mmol^{-1} × cm^{-1}]

It follows for urea:

$$c = \frac{3.040 \times 60.06}{\varepsilon \times 1.00 \times 0.100 \times 2 \times 1000} \times \Delta A_{urea} = \frac{0.9129}{\varepsilon} \times \Delta A_{urea}\ [\text{g urea/l sample solution}]$$

for ammonia:

$$c = \frac{3.040 \times 17.03}{\varepsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A_{ammonia} = \frac{0.5177}{\varepsilon} \times \Delta A_{ammonia}\ [\text{g ammonia/l sample solution}]$$

If the sample has been diluted on preparation, the result must be multiplied by the dilution factor F.

1 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm **or** 334 nm.
2 If desired, disposable cuvettes may be used instead of glass cuvettes.
3 See instructions for performance of assay

* For simplification of the assay performance it is also possible to pipette directly 1.000 ml of solution 1 into the cuvette and add 1 tablet from bottle 2. After dissolution of the tablet with the aid of a spatula continue working as described in the procedure. The difference in volume of approx. 1% (increase of volume by 1 tablet per 3.040 ml assay volume) has to be taken into account in the calculation by multiplication of the result with 1.01.

** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

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When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{urea}} = \frac{c_{\text{urea}}\ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [\text{g/100 g}]$$

$$\text{Content}_{\text{ammonia}} = \frac{c_{\text{ammonia}}\ [\text{g/l sample solution}]}{\text{weight}_{\text{sample}}\ \text{in g/l sample solution}} \times 100\ [\text{g/100 g}]$$

1. Instructions for performance of assay

The amount of urea (ammonia) present in the assay has to be between 0.3 µg and 14 µg (0.2 µg and 8 µg). In order to get a sufficient absorbance difference, the sample solution is diluted to yield an urea (ammonia) concentration between 0.02 and 0.14 g/l (0.01 and 0.08 g/l).

Dilution table

Estimated amount of urea (ammonia) per liter	Dilution with water	Dilution factor F
< 0.14 g (< 0.08 g)	-	1
0.14-1.4 g (0.08-0.8 g)	1 + 9	10
1.4-14 g (0.8-8.0 g)	1 + 99	100

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) *or* the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical information

2.1 Use only freshly distilled water for the assay.

2.2 Work in an atmosphere free from ammonia (ban smoking in the laboratory).

3. Specificity (Ref. 1)

The method is specific for urea and ammonia.

In the analysis of commercial urea and ammonium sulfate results of approx. 100% have to be expected.

4. Sensitivity and detection limit (Ref. 1.4)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml and measurement at 340 of an ammonia concentration of 0.02 mg/l sample solution, resp. of an urea concentration of 0.04 mg/l (if v = 0.100 ml, this corresponds to 0.4 mg ammonia/l, resp. 0.8 mg urea/l sample solution).

The detection limit of 0.08 mg ammonia/l, resp. 0.15 mg urea/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume v = 2.000 ml.

5. Linearity

Linearity of the determination exists from approx. 0.2 µg ammonia/ assay (0.08 mg ammonia/l sample solution; sample volume v = 2.000 ml) to 8 µg ammonia/assay (0.08 g ammonia/l sample solution; sample volume v = 0.100 ml), resp. from 0.3 µg urea/assay (0.15 mg urea/l sample solution; sample volume v = 2.000 ml) to 14 µg urea/ assay (0.14 g urea/l sample solution; sample volume v = 0.100 ml).

6. Precision

Ammonia:

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to an ammonia concentration of approx. 0.4-1 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.004-0.01 g/100 g can be expected.)

The following data for the determination of ammonia have been published in the literature:

CV = 1.6 %	(plasma)	(Ref. 1.2)
CV = 0.88-1.16 %	(ammonium chloride solutions)	(Ref. 1.4)
CV = 0.34 %	(ammonium chloride solutions)	
CV = 0.36-0.96 %	(meat samples)	(Ref. 3.2)

Urea:

In a double determination using one sample solution, a difference of 0.005 to 0.015 absorbance units may occur. With a sample volume of v = 0.100 ml and measurement at 340 nm, this corresponds to an urea concentration of approx. 0.7-2 mg/l. (If the sample is diluted during sample preparation, the result has to be multiplied by the dilution factor F. If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.007- 0.02 g/100 g can be expected.)

The following data for the determination of urea have been published in the literature:

CV = 2.7 %	(serum)	(Ref. 1.1)
CV = 3 %	(serum)	(Ref. 1.3)

Analysis of swimming-pool water: (Ref. 3.7)

x = 0.611 mg/l	r = 0.1854 mg/l	$s_{(r)} = \pm$ 0.066 mg/l
	R= 0.2145 mg/l	$s_{(R)} = \pm$ 0.076 mg/l
x = 2.323 mg/l	r = 0.1247 mg/l	$s_{(r)} = \pm$ 0.044 mg/l
	R= 0.1883 mg/l	$s_{(R)} = \pm$ 0.067 mg/l
x = 5.749 mg/l	r = 0.0707 mg/l	$s_{(r)} = \pm$ 0.025 mg/l
	R= 0.1707 mg/l	$s_{(R)} = \pm$ 0.060 mg/l

7. Interference/sources of error

During protein precipitation with perchloric acid which is to be carried out in foodstuffs, protein fragments are occasionally obtained. These protein fragments are kept in solution and may gradually form ammonia in alkaline buffer systems leading to creep reactions. This formation of ammonia is very low and can be differentiated and calculated from the ammonia content of the sample by extrapolation of the absorbance A_2 to the time of addition of solution 4 (GlDH).

The common ingredients of foodstuffs do not interfere with the assay of urea and ammonia. Only high concentrations of tannins in fruit juices may cause an inhibiton of the GlDH reaction. Fruit juices should therefore always be treated with PVPP.

As high concentrations of heavy metals cause turbidity, they make a reliable determination of ammonia difficult. In most cases high concentrations of metal ions can be removed as hydroxides by alkalization of the sample solution (pH > 7.5).

Sodium thiosulfate, occasionally added to samples of swimming-pool water, does not interfere with the assay up to 1 mg per assay.

8. Recognizing interference during the assay procedure

8.1 If the conversion of urea and ammonia has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding urea and/or ammonium chloride or ammonium sulfate (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample **and** assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of urea and ammonia are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:
Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml;
Filter **turbid solutions**;
Degas **samples containing carbon dioxide** (e.g. by filtration);
Adjust **acid samples** to pH 7-8 by adding sodium or potassium hydroxide solution;
Adjust **acid and weakly colored samples** to approx pH 7-8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;
Treat **"strongly colored" samples** that are used undiluted or with a higher sample volume with polyvinylpolypyrrolidone (PVPP) - (e.g. 2.5-5 g/100 ml);
Crush or homogenize **solid or semi-solid samples,** extract with water or dissolve in water and filter if necessary;
Deproteinize **samples containing protein** with perchloric acid or with trichloroacetic acid;
Extract **samples containing fat** with hot water (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter;
Break up **emulsions** with trichloroacetic acid.

Important note

The Carrez-clarification should not be used in the sample preparation for urea / ammonia determination due to a too low recovery rate (adsorption of urea/ammonia).

11. Application examples

Determination of ammonia in fruit juices
Add 0.5-1.0 g wet polyvinylpolypyrrolidone (PVPP) to 10 ml fruit juice (clear, turbid or colored juices) - when the sample volume is increased, neutralize, if necessary, and fill up to 20 ml with water - in a beaker and stir for 1 min (magnetic stirrer). Filter sample solution immediately and use it for the assay. In the assay, only "blank ammonia" and "sample ammonia" are to be measured.

Determination of urea and ammonia in water (swimming-pool water)
Dilute sample solution according to the dilution table or use up to v = 2.000 ml sample volume for the assay.

Determination of urea in milk
Mix 1 ml milk with 4 ml trichloroacetic acid (0.3 M). After approx. 5 min centrifuge for separation of the precipitate (for 3 min, ca. 4000 rpm). Use 0.100 ml of the supernatant clear solution for the assay.

Determination of ammonia in milk
Mix 1 ml milk with 4 ml trichloroacetic acid (0.3 M). After approx. 5 min centrifuge for separation of the precipitate. Decant the supernatant and neutralize with KOH (10 M) (dilution factor can be neglected due to the high concentration of KOH), filter and use 1.000-2.000 ml sample solution for the assay.
In the assay, only "blank ammonia" and "sample ammonia" are to be measured.

Determination of ammonia in bakery products
Accurately weigh approx. 10 g of the minced sample into a homogenizer beaker, add approx. 20 ml perchloric acid (1 M) and homogenize for approx. 2 min. Proceed as stated under "meat and meat products". Use at most 1.000 ml for the assay.
In the assay, only "blank ammonia" and "sample ammonia" are to be measured.

Determination of urea and ammonia in meat and meat products
Accurately weigh approx. 5 g of the homogenized sample (from a sample of 100 g, that has been ground and homogeneously mixed in a mixer) into a homogenizer beaker, add approx. 20 ml perchloric acid (1 M) and homogenize for approx. 2 min. Transfer the contents quantitatively with approx. 40 ml water into a beaker. Adjust to pH 7.0 (< 7.5) first with potassium hydroxide (5 M) and then exactly with potassium hydroxide (2 M). Transfer the contents quantitatively with water into a 100 ml volumetric flask, fill up to the mark with water, whereby it must be taken care that the fatty layer is above the mark and the aqueous layer is **at** the mark.

For separation of fat and for precipitation of the potassium perchlorate refrigerate for 20 min. Afterwards filter. Discard the first few ml. Use the clear, possibly slightly turbid solution for the assay.

Calculate of the amount of urea and ammonia according to the aforementioned calculation formula, whereby it must be multiplied with the volume displacement factor K = 0.98.

12. Further applications

The method may also be used in the examination of fertilizers, pharmaceuticals, cosmetics, paper (Ref. 2.1) and in research when analyzing biological samples. For details of sampling, treatment and stability of the sample see Ref. 1.1-1.4.

Examples:

12.1 Determination of urea and ammonia in fertilizers

Grind approx. 10 g of the sample and mix thoroughly. Accurately weigh approx. 100 mg of the homogeneous material into a 100 ml beaker and add approx. 50 to 60 ml water. Adjust to pH 7-8 with diluted hydrochloric acid (1 M) or in the case of acidic fertilizer with diluted sodium hydroxide (1 M). Warm on a heatable magnetic stirrer for approx. 10 min to 60-70°C. Allow to cool, transfer quantitatively into a 100 ml volumetric flask and fill up to the mark with water. Mix the solution and filter, if necessary.
Use 0.100 ml of the clear solution diluted, if necessary, for the assay.

12.2 Determination of nitrogen after Kjeldahl-digestion

The determination of total nitrogen can be obtained via the ammonia determination in a sample mineralized according to the Kjeldahl-method. Normally, the samples are to be incinerated wet (sulfuric acid). The ammonia, formed from nitrogen, is determined according to the procedure as follows.

Accurately weigh approx. 2 g of the ground and homogenized sample into a 100 ml Kjeldahl-flask, add 20 ml sulfuric acid (specific gravity = 1.84 g/ml) and approx. 30 mg catalyst mixture (e.g., acc. to Wieninger) or one Kjeldahl tablet, heat for approx. 2-3 h until the sample is disintegrated (yellowish or blue-greenish solution). Allow the sample to cool and carefully (protective glasses) transfer quantitatively into a beaker filled with 600 ml ice-cold water, while stirring all the time (magnetic stirrer, icebath). Neutralize with approx. 60 ml KOH (10 M) (pH 6-8). Transfer the neutralized solution quantitatively into a 1 l volumetric flask, fill up to the mark with water and mix. If necessary, filter the mixture (sometimes necessary after disintegration with Kjeldahl tablets); discard the first few ml. Use the solution diluted, if necessary, for the assay.

Calculation:

Nitrogen content of the sample (in %)

$$= \frac{\Delta A \times V \times MW \times 100}{\varepsilon \times d \times v \times 1000 \times \text{amount weighed [g]}} =$$

$$= \frac{\Delta A \times 3.04 \times 14.01 \times 100}{\varepsilon \times 1.00 \times 0.100 \times 1000 \times \text{amount weighed [g]}}$$

12.3 Determination of urea and ammonia in fermentation samples and cell culture media

Place the sample (after centrifugation, if necessary) in a waterbath at 80°C for 15 min to stop enzymatic reactions. Centrifuge and use the supernatant (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinization can be carried out with perchloric acid. See the above-mentioned examples.

Homogenize gelatinous agar media with water and treat further as described.

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Urea assay control solution

The assay control solution serves as a control for the enzymatic determination of urea in foodstuffs and other materials.

Reagents

Urea, AR grade

Preparation of the assay control solution

Accurately weigh approx. 140 mg urea to the nearest 0.1 mg into a 1000 ml volumetric flask, fill up to the mark with redist. water, and mix thoroughly.

Prepare assay control solution freshly before use. The assay control solution may be frozen in portions.

Application:

1. Addition of the assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay. (The measurement fo the assay control solution is not necessary for calculating the results.)

2. Restart of the reaction, quantitatively:
After completion of the reaction with sample solution and measuring of A_2, add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 20 min). Calculate the concentration from the difference of (A_2-A_3) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the assay control solution, the result differs insignificantly from the result got according to pt. 1.

3. Internal standard:
The assay control solution can be used as an internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
reaction mixture 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
solution 3	0.020 ml	0.020 ml	0.020 ml	0.020 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml
Mix, and read absorbances of the solutions (A_1) after approx. 5 min. Continue as described in the pipetting scheme under “Procedure” . Follow the instructions given under “Instructions for performance of assay” and the footnotes.				

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100\ [\%]$$

R-BIOPHARM AG
Landwehrstr. 54
D-64293 Darmstadt
Telefon + 49 61 51 / 81 02-0
Fax + 49 61 51 / 81 02-20
www.r-biopharm.com

Roche