Megazyme

www.megazyme.com

L-ASPARAGINE/ L-GLUTAMINE/ AMMONIA (*RAPID*)

ASSAY PROCEDURE

K-ASNAM 04/18

(*50 Assays of each per kit)

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

INTRODUCTION:

The simple and rapid measurement of L-asparagine and ammonia together has recently become of great significance with respect to two major applications:

1. Cell Culture: L-Asparagine is an essential component of certain cell culture media. However, the incorporation of this amino acid into growth media presents two major problems; firstly, L-asparagine is labile, and spontaneously breaks down to L-aspartate and free ammonium ions. Secondly, the released ammonium ions are very toxic to the cells. To overcome these issues, L-asparagine is added just prior to use and its concentration along with that of ammonia is frequently monitored during culturing.

This kit (**K-ASNAM**), based on the use of advanced recombinant enzymes, is very rapid (~ 20 min), and also measures both L-asparagine and ammonia in a very simple format. A value for L-glutamine is also produced as part of the method that may also be of significance in cell culture applications. Both manual (see page 6, "A") and microplate (see page 9, "B") formats are described. Other rapid tissue culture test kits are also available from Megazyme for ammonia (**K-AMIAR**), L-glutamine/ammonia (**K-GLNAM**), D-glucose (**K-GLUHK** or **K-GLUC**), and L-lactic acid (**K-LATE**).

2. Acrylamide Precursors: It is now well known that when L-asparagine, ammonium ions, D-fructose and/or D-glucose are heated above approximately 160°C, significant levels of the carcinogenic compound acrylamide are formed, with obvious concerns for human health. Food products affected include potato crisps and chips, roasted potatoes and other fried, toasted or roasted foods, such as bakery goods, breakfast cereals and coffee. Asparaginase, an enzyme that converts L-asparagine into L-aspartic acid, thus has potential applications in this area for reducing acrylamide levels. However, if asparaginase treatment is adopted, it will be necessary to confirm that the free L-asparagine has been successfully converted to L-aspartic acid. This kit (**K-ASNAM**) is ideal for this application and, along with the Megazyme D-fructose/D-glucose kit (**K-FRUGL**), enables the concentrations of all four key acrylamide precursors to be determined both simply and cost effectively.

This kit is also suitable for the analysis of L-asparagine, L-glutamine and ammonia in a wide range of other samples. Most notably, this method first converts/quantifies L-glutamine, an amino acid that would otherwise lead to interference due to the low but significant action of asparaginase on this compound.

PRINCIPLE:

Determination of L-asparagine, L-glutamine and ammonia takes place in three simple and rapid steps; L-glutamine is first converted by a large excess of glutaminase, into L-glutamate and ammonium ions (NH_4^+) (1).

$$\text{(1) L-Glutamine} + H_2O \xrightarrow{\text{(glutaminase)}} \text{L-glutamate} + NH_4^+$$

Then, in the presence of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and glutamate dehydrogenase (GlDH), the ammonia from the sample, and also that formed by reaction (1), reacts with 2-oxoglutarate to form L-glutamate and $NADP^+$ (2).

$$\text{(2) } NH_4^+ + \text{2-oxoglutarate} + \text{NADPH} \xrightarrow{\text{(GlDH)}} \text{L-glutamate} + NADP^+ + H_2O$$

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

In the final reaction, L-asparagine is rapidly hydrolysed to L-aspartate and ammonium ions by asparaginase (3).

$$\text{(3) L-Asparagine} + H_2O \xrightarrow{\text{(asparaginase)}} \text{L-aspartate} + NH_4^+$$

The ammonium ions liberated react according to (2) leading to a further fall in absorbance that is stoichiometric with the amount of L-asparagine.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for L-asparagine, L-glutamine and free ammonium ions. The D-isomers do not react.

The assay is linear over the range of 0.2 to 7.0 µg of ammonia, or 0.5 to 50 µg of L-asparagine or L-glutamine 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 1.00 mL, this corresponds to an ammonia concentration of approx. 0.316 to 0.633, L-glutamine concentration of approx. 0.271 to 0.5427, or an L-asparagine concentration of approx. 0.247 to 0.4949 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 quantitative deamination of L-asparagine and L-glutamine was achieved (reactions 3 and 1, respectively), within the time specified in the assay (i.e. approx. 5 min), it can be generally concluded that no interference has occurred. Interference of the indicator reaction (2) can be 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 L-asparagine and/or L-glutamine to the sample in the initial extraction steps.

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 each of L-asparagine, L-glutamine and ammonia are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer (11 mL, pH 4.9) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2: (x2)	Buffer (25.5 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 3: (x2) NADPH. Lyophilised powder.
Stable for > 5 years below -10°C.

Bottle 4: Glutaminase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 5: Glutamate dehydrogenase suspension (2.2 mL).
Stable for > 2 years at 4°C.

Bottle 6: Asparaginase suspension (1.1 mL).
Stable for > 2 years at 4°C.

Bottle 7: Ammonia standard solution (5 mL, 0.04 mg/mL) in 0.02% sodium azide.
Stable for > 2 years at 4°C.

Bottle 8: L-Asparagine control powder (~ 2 g).
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

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

3. Dissolve the contents of one of bottle 3 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).

4, 5 & 6. Use the contents of bottles 4, 5 and 6 as supplied. Before opening for the first time, shake the bottles to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottles in an upright position.
Stable for > 2 years at 4°C.

7. Use the contents of bottle 7 as supplied.
Stable for > 2 years at 4°C.

8. Accurately weigh approx. 0.300 g of L-asparagine into a 1 L volumetric flask, fill to the mark with distilled water and mix thoroughly.
Stable for ~ 3 months below -10°C.

NOTE: The L-asparagine and ammonia standard solutions are 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 L-asparagine, L-glutamine and ammonia are determined directly from the extinction coefficient of NADPH (page 7).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 500 mL).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (200 µL and 1000 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5 mL Combitip® [to dispense 0.2 mL aliquots of buffer (solution 1)].
   - with 25 mL Combitip® (to dispense 1.5 mL aliquots of distilled water and 0.5 mL aliquots of solution 3).
5. Analytical balance.
6. Spectrophotometer or plate reader 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 FORMAT:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.34 mL (ammonia and L-glutamine)
2.36 mL (L-asparagine)
Sample solution: 0.2-7.0 µg of ammonia per cuvette
or 0.50-50 µg of L-asparagine per cuvette
or 0.5-50 µg of L-glutamine per cuvette
(in 0.1-1.0 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Ammonia		GLN/ASN	
	Blank	Sample	Blank	Sample
solution 1 (buffer, 4.9)	-	-	0.20 mL	0.20 mL
sample solution	-	0.10 mL	-	0.10 mL
suspension 4 (Glutaminase)	-	-	0.02 mL	0.02 mL
Mix* and incubate for 5 min at room temperature. **Then add:**				
distilled water (at ~ 25°C)	1.82 mL	1.72 mL	1.60 mL	1.50 mL
solution 2 (buffer, 8.0)	0.30 mL	0.30 mL	0.30 mL	0.30 mL
solution 3 (NADPH)	0.20 mL	0.20 mL	0.20 mL	0.20 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 5 min. Then start the reaction by addition of:				
suspension 5 (GIDH)	0.02 mL	0.02 mL	0.02 mL	0.02 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 remain the same. **Then add:**				
suspension 6 (Asparaginase)	-	-	0.02 mL	0.02 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®.

CALCULATION:

Determine the absorbance differences (A_1-A_2) and (A_2-A_3) for both blanks and samples. Subtract the absorbance difference of the blank from the absorbance difference of the corresponding sample, thereby obtaining the change in absorbance (ΔA) resulting from the analyte in question, as follows:

Determination of ammonia:

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

Determination of L-glutamine:

To calculate $\Delta A_{L\text{-}glutamine}$, it is first necessary to calculate $\Delta A_{(L\text{-}glutamine + ammonia)}$, in order to account for the free ammonium ions in the sample:

$$\Delta A_{(L\text{-}glutamine + ammonia)} = (A_1\text{-}A_2)_{GLN / ASN\ sample} - (A_1\text{-}A_2)_{GLN / ASN\ blank}$$

Then:

$$\Delta A_{L\text{-}glutamine} = \Delta A_{(L\text{-}glutamine + ammonia)} - \Delta A_{ammonia}$$

Determination of L-asparagine:

$$\Delta A_{L\text{-}asparagine} = (A_2\text{-}A_3)_{GLN / ASN\ sample} - (A_2\text{-}A_3)_{GLN / ASN\ blank}$$

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

The concentration of ammonia, L-glutamine and L-asparagine 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 analyte [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:

$$c = \frac{2.34 \times 17.03}{6300 \times 1.0 \times 0.10} \times \Delta A_{ammonia} \quad [g/L]$$

$$= 0.06325 \times \Delta A_{ammonia} \quad [g/L]$$

It follows for L-glutamine:

$$c = \frac{2.34 \times 146.1}{6300 \times 1.0 \times 0.10} \times \Delta A_{L\text{-}glutamine} \quad [g/L]$$

$$= 0.5427 \times \Delta A_{L\text{-}glutamine} \quad [g/L]$$

It follows for L-asparagine:

$$c = \frac{2.36 \times 132.1}{6300 \times 1.0 \times 0.10} \times \Delta A_{L\text{-}asparagine} \quad [g/L]$$

$$= 0.4949 \times \Delta A_{L\text{-}asparagine} \quad [g/L]$$

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

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

Content of L-glutamine

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

Content of L-asparagine

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

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. MICROPLATE FORMAT:

Wavelength: 340 nm
Microplate: 96 well (e.g. clear, flat-bottomed, polypropylene)
Temperature: ~ 25°C
Final volume: 234 µL (ammonia and L-glutamine)
236 µL (L-asparagine)
Sample solution: 0.020-0.70 µg of ammonia per well
or 0.05-5.0 µg of L-asparagine per well
or 0.05-5.0 µg of L-glutamine per well
(in 10 µL sample volume)

Pipette into cuvettes	Ammonia		GLN/ASN	
	Blank	Sample	Blank	Sample
solution 1 (buffer, 4.9)	-	-	20 µL	20 µL
sample solution	-	10 µL*	-	10 µL
suspension 4 (Glutaminase)	-	-	2 µL*	2 µL*
Mix** and incubate for 5 min at room temperature. **Then add:**				
distilled water (at ~ 25°C)	182 µL	172 µL	160 µL	150 µL
solution 2 (buffer, 8.0)	30 µL	30 µL	30 µL	30 µL
solution 3 (NADPH)	20 µL	20 µL	20 µL	20 µL
Mix** and read the absorbances of the solutions (A_1) after approx. 5 min. Then start the reaction by addition of:				
suspension 5 (GlDH)	2 µL*	2 µL*	2 µL*	2 µL*
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 remain the same. **Then add:**				
suspension 6 (Asparaginase)	-	-	2 µL*	2 µL*
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.				

* If preferred, dilute sufficient enzyme for the set of assays 10-fold with distilled water and add 20 µL. Reduce the amount of water appropriately (i.e. by 18 µL) to maintain the same final volume.

** for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using pipettor set at 50-100 µL volume).

CALCULATION:

Calculations can be performed as described on pages 7 and 8, after appropriate path-length adjustment to 10 mm. This can either be performed automatically by the plate reader or after manual determination of the true path-length (i.e. by simply performing a "manual" format assay of the control ammonia solution, and comparing the absorbance change to that of a reaction performed according to the "microplate" format). Alternatively, a standard curve can be used.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of L-glutamine and L-asparagine present in the cuvette (i.e. in the 0.10 mL of sample being analysed) should range between 0.05 and 50 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.005 and 0.50 g/L.

Dilution Table

Estimated concentration of L-glutamine and L-asparagine (g/L)	Dilution with water	Dilution factor (F)
< 0.50	No dilution required	1
0.50-5.0	1 + 9	10
5.0-50	1 + 99	100
> 50	1 + 999	1000

If the value of $\Delta A_{ammonia}$, $\Delta A_{L\text{-glutamine}}$ or $\Delta A_{L\text{-asparagine}}$ 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 1.00 mL, making sure that the sum of the sample and distilled water components in the cuvette is either 1.60 mL (for L-glutamine) or 1.82 mL (for ammonia) 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 can be used as an alternative (see specific example).

3. General considerations.

NB - these considerations are for the analysis of complex samples, such as foodstuffs and beverages. They are not necessary in the analysis of cell culture media/supernatants; see "Sample preparation example A".

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) 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.

(c) Coloured samples: an additional sample blank, i.e. sample with no GlDH, may be necessary in the case of coloured samples.

(d) 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.

(e) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(f) 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 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.

(g) 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.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of ammonia, L-glutamine and L-asparagine in cell culture media/supernatants.

In general, the concentration of ammonia, L-glutamine and L-asparagine in liquid cell culture media/supernatants can be determined without any sample treatment (except clarification by centrifugation/filtering or dilution according to the dilution table, if necessary).

Typically, no clarification or dilution is required, and a sample volume of 0.1 mL is satisfactory.

(b) Determination of ammonia, L-glutamine and L-asparagine in powdered dietary supplements.

In general, the concentration of ammonia, L-asparagine and L-glutamine in dietary supplements, such as pharmaceutical grade L-glutamine, can be determined as follows: accurately weigh approx. 5 g of representative material into a 100 mL volumetric flask. After addition of approx. 60 mL of distilled water, stir the contents until fully dissolved or suspended, and fill up to the mark with distilled water. Mix and, if necessary, filter through Whatman No. 1 filter paper. Use the clear filtrate, with dilution according to the dilution table if necessary. *Typically, for pharmaceutical grade L-glutamine, a further dilution of 1:100 and sample volume of 0.1 mL are satisfactory.*

(c) Determination of ammonia, L-glutamine and L-asparagine in sports nutrition and bakery products (e.g. snack bars).

Homogenise approx. 10 g of material and accurately weigh approx. 2 g into a 100 mL volumetric flask. Add 60 mL of distilled water, and incubate at 60°C for 5 min, or until fully suspended. Allow to equilibrate to room temperature and fill to the mark with distilled water (ensuring any fat containing layer is "above" the mark, and the aqueous layer is "at" the mark). Filter, discarding the first 3-5 mL and use the filtrate for the assay. *Typically, no further dilution is required and a sample volume of 0.1 mL is satisfactory.*

(d) Determination of ammonia, L-glutamine and L-asparagine in fruit and vegetable products/preparations (e.g. potato juice).

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 any 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 any 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 0.1 mL is satisfactory.*

REFERENCE:

Lund, P. (1990). L-Glutamine and L-Glutamate. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VIII**, pp. 357-363, VCH Publishers (UK) Ltd., Cambridge, UK.

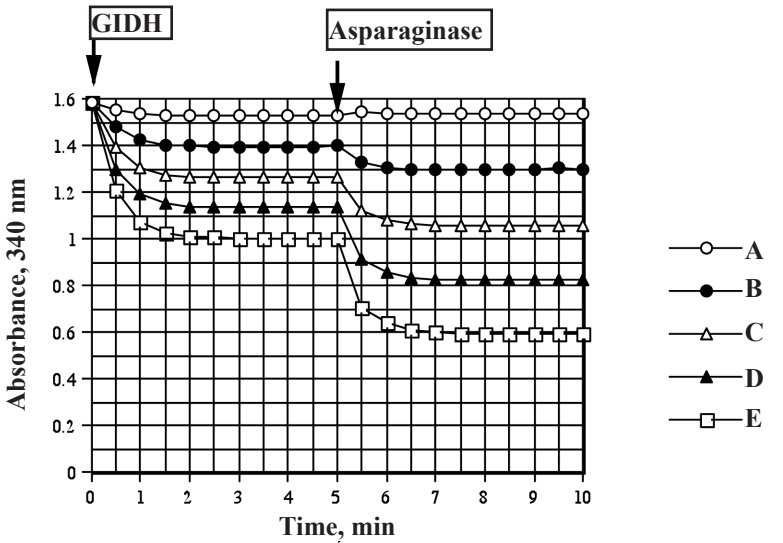

Figure 1. Decrease in absorbance at 340 nm on incubation of ammonia/L-asparagine standard mixtures with glutamate dehydrogenase followed by asparaginase in the presence of NADPH. **A**. 0 µg ammonia, 0 µg L-asparagine; **B**. 0.8 µg ammonia, 5.3 µg L-asparagine; **C**. 1.6 µg ammonia, 10.6 µg L-asparagine; **D**. 2.4 µg ammonia, 15.8 µg L-asparagine; **E**. 3.2 µg ammonia, 21.1 µg L-asparagine.

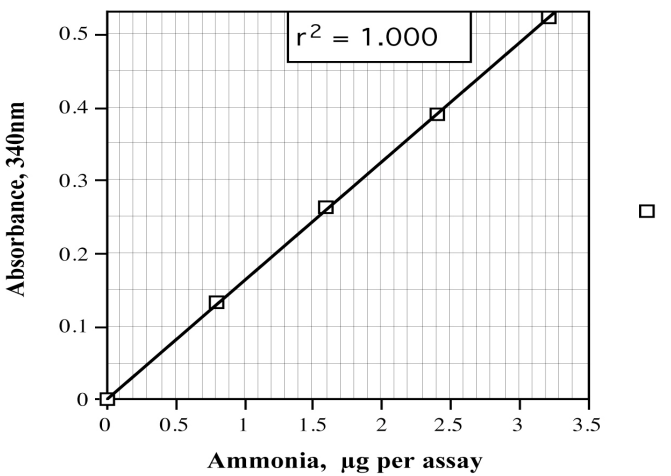

Figure 2. Calibration curve demonstrating the linearity of **K-ASNAM** in ammonia determination. The reactions used to generate this calibration curve were performed at room temperature for 5 min using a 10 mm path-length cuvette.

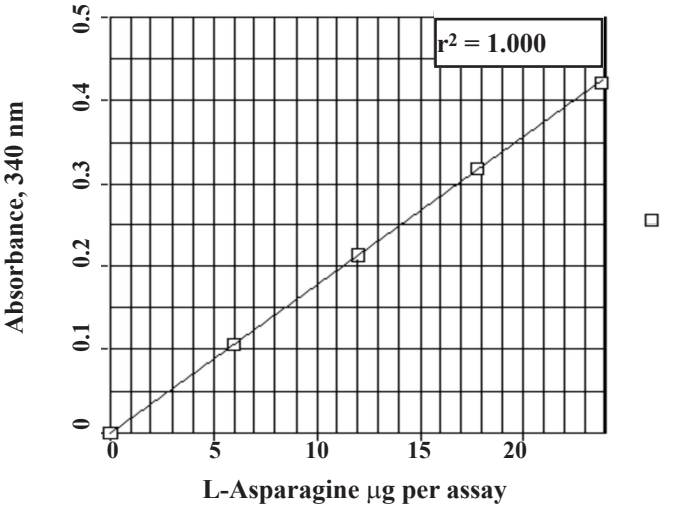

Figure 3. Calibration curve demonstrating the linearity of **K-ASNAM** in L-asparagine determination. The reactions used to generate this calibration curve were performed at room temperature for 5 min, using a 10 mm path-length cuvette.

NOTES:

Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com