Megazyme

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L-GLUTAMIC ACID (L-GLUTAMATE)

ASSAY PROCEDURE

K-GLUT 04/18

(*60 Assays per Kit) or
(700 Auto-Analyser Assays per Kit) or
(600 Microplate Assays per Kit)

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

INTRODUCTION:

L-Glutamic acid (L-glutamate), one of the 20 common amino acids, occurs naturally in foods such as cheese, milk, meat, fish, corn, tomatoes, mushrooms, soybean and sugar beet. In its free form, L-glutamic acid is the major flavour enhancing component of foods with a meaty/savoury taste. The role played by L-glutamic acid in the palatability of foods is exploited by either traditional cooking methods that release free L-glutamic acid, or by supplementation with monosodium glutamate (MSG). Excessive use of MSG as a food additive (E621) in processed foods results in the symptoms of "Chinese Restaurant Syndrome" (CRS), where palpitations, severe headaches, nausea, wheezing and increased body temperature are thought to result from the neuro-excitory properties of free L-glutamic acid. Onset of CRS is fast, only 30-60 min after ingestion, and symptoms generally subside after approximately 2-3 hours. Around 15% of Americans are sensitive to MSG.

PRINCIPLE:

L-Glutamic acid is oxidised by nicotinamide-adenine dinucleotide (NAD^+) in the presence of glutamate dehydrogenase (GlDH), leading to the formation of 2-oxoglutarate, reduced nicotinamide-adenine dinucleotide (NADH) and ammonium ions (NH_4^+) (1).

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

However, as the equilibrium of this deamination reaction lies markedly in the favour of the reactants, a further reaction catalysed by diaphorase is required, in which NADH reduces iodonitrotetrazolium chloride (INT) to an INT-formazan product, leading to a rapid and quantitative conversion of L-glutamic acid (2).

$$\text{(2)}\quad \text{INT} + NADH + H^+ \xrightarrow{\text{(diaphorase)}} NAD^+ + \text{INT-formazan}$$

The amount of INT-formazan formed in this reaction is stoichiometric with the amount of L-glutamic acid. It is the INT-formazan which is measured by the increase in absorbance at 492 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for L-glutamic acid. D-Glutamic acid, L-glutamine and L-aspartic acid do not react.

The smallest differentiating absorbance for the assay is 0.005 absorbance units. This corresponds to 0.054 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.214 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 0.4 to 20 µg of L-glutamic acid 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 0.10 mL, this corresponds to a L-glutamic acid concentration of approx. 1.07 to 2.14 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:

High concentrations of L-ascorbic acid, cysteine or sulphite interfere with the assay as they react with INT causing a non-enzymic "creep" rate. These compounds should be removed by treating the sample with H_2O_2 and alkali as follows:

Weigh or pipette sample, diluted if necessary, into a 50 mL volumetric flask. Add water to a volume of approx. 40 mL, and then add 1 mL of 2 M KOH and 0.01 mL of H_2O_2 (30% v/v). Incubate the solution for 10 min at approx. 70°C. Cool to 20-25°C and adjust to approx. pH 8.6 with 1 M H_2SO_4. Fill to the mark with distilled water, mix, filter and use the solution for the assay.

As NH_4^+ ions are a product of the GlDH reaction [see (1) on page 1], significant quantities present in a sample will lead to reduced rates of L-glutamic acid conversion. It is therefore recommended that if present at > 5 mM, NH_4^+ ions be removed as follows: increase the pH of the sample to approx. 9.0 with NaOH and incubate at 60°C for 30 min. The ammonium hydroxide formed decomposes to NH_3 gas and water, the former of which is rapidly lost from the solution.

If the conversion of L-glutamic acid has been completed within the time specified in the assay (approx. 8-10 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding L-glutamic acid (approx. 10 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase 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-glutamic acid 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 60 assays in manual format (or 700 assays in auto-analyser format or 600 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (35 mL, pH 8.6) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: (x2) NAD^+ plus INT.
Stable for > 2 years at 4°C.

Bottle 3: Diaphorase suspension (3.1 mL).
Stable for > 2 years at 4°C.

Bottle 4: Glutamate dehydrogenase solution (3.1 mL).
Stable for > 2 years below -10°C.

Bottle 5: L-Glutamic acid standard solution
(5 mL, 0.10 mg/mL) in 0.02% (w/v) sodium azide.
Stable for > 2 years at 4°C.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

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

2. Dissolve the contents of one of bottle 2 in 6.5 mL of distilled water. Divide into appropriately sized aliquots and store in a dark container below -10°C between use and keep cool during use if possible. Once dissolved, the reagent is stable for > 12 months below -10°C.

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that

may have settled on the stopper. 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. Use the contents of bottle 5 as supplied.
Stable for > 2 years at 4°C.

NOTE: The L-glutamic acid 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 L-glutamic acid is determined directly from the extinction coefficient of INT-formazan (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 and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of NAD^+/INT solution).
   - with 12.5 mL Combitip® [to dispense 0.5 mL aliquots of buffer (bottle 1)].
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 492 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 ASSAY PROCEDURE:

Wavelength: 492 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.90 mL
Sample solution: 0.4-20 µg of L-glutamic acid 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 mix)	0.50 mL	0.50 mL
solution 2 (NAD^+/INT)	0.20 mL	0.20 mL
suspension 3 (diaphorase)	0.05 mL	0.05 mL
Mix* and read the absorbances of the solutions (A_1) after approx. 2 min. Repeat the measurement after another 2 min. If a change in absorbance greater than 0.010 is observed, the sample must be treated to remove reducing substances (see "interference" section on page 2). Start the reactions immediately by addition of:		
solution 4 (GIDH)	0.05 mL	0.05 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 8-10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances either plateau or increase 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).

NOTE: INT and the reaction system containing INT are sensitive to light. Consequently, reactions must be performed in the dark (e.g. in the spectrophotometer cuvette compartment with the photometer lid closed).

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{L-glutamic acid}}$.

The value of $\Delta A_{\text{L-glutamic acid}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of L-glutamic acid can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{\text{L-glutamic acid}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of L-glutamic acid [g/mol]
ε = extinction coefficient of INT-formazan at 492 nm
= 19900 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for L-glutamic acid:

$$c = \frac{2.90 \times 147.13}{19900 \times 1.0 \times 0.10} \times \Delta A_{\text{L-glutamic acid}} \quad [g/L]$$

$$= 0.2144 \times \Delta A_{\text{L-glutamic acid}} \quad [g/L]$$

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 L-glutamic acid

$$= \frac{c_{\text{L-glutamic acid}}\ [g/L \text{ sample solution}]}{\text{weight}_{\text{sample}}\ [g/L \text{ sample solution}]} \times 100 \quad [g/100\ g]$$

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. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for L-glutamic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of L-glutamic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
bottle 1 (buffer)	6.0 mL
bottle 2 (NAD^+/INT)	2.4 mL (after adding 6.5 mL of H_2O to bottle 2)
bottle 3 (diaphorase)	0.6 mL
distilled water	20.6 mL
Total volume	29.6 mL

Preparation of R2:

Component	Volume
bottle 4 (GlDH)	0.6 mL
distilled water	3.1 mL
Total volume	3.7 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 10 min at 37°C
Wavelength:	492 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.16 g/L of L-glutamic acid using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for L-glutamic acid can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of L-glutamic acid **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 492 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.290 mL
Linearity: 0.1-2 µg of L-glutamic acid per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.050 mL	0.050 mL	0.050 mL
solution 2 (NAD^+/INT)	0.020 mL	0.020 mL	0.020 mL
solution 3 (diaphorase)	0.005 mL	0.005 mL	0.005 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 2 min and start the reactions by addition of:			
solution 4 (GlDH)	0.005 mL	0.005 mL	0.005 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 8-10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances either plateau or increase constantly over 2 min.			

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

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of L-glutamic acid present in the cuvette (i.e. in the 0.1 mL of sample being analysed), should range between 0.4 and 20 µg. The sample solution must therefore be diluted sufficiently to yield a L-glutamic acid concentration between 0.004 and 0.2 g/L.

Dilution Table

Estimated concentration of L-glutamic acid (g/L)	Dilution with water	Dilution factor (F)
< 0.20	No dilution required	1
0.2-2.0	1 + 9	10
2.0-20	1 + 99	100
> 20	1 + 999	1000

If the value of $\Delta A_{\text{L-glutamic acid}}$ 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:

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

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. 8.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 8.6 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. Adjust to 20°C 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. Alternatively, clarify with Carrez reagents.

(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 Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of L-glutamic acid in soy sauce.

The L-glutamic acid concentration of soy sauce can generally be determined without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:500 and sample volume of 0.2 mL are satisfactory.*

(b) Determination of L-glutamic acid in meat extracts, soups and bouillon concentrates.

Accurately weigh approx. 1 g of representative material (for powdered samples) or 1 mL of liquid (in the case of suspensions) into a 100 mL volumetric flask. Add 70 mL of distilled water and incubate at 70°C for 10 min to extract the L-glutamic acid. Allow to cool and adjust the volume to the mark with distilled water. Filter the solution through Whatman No. 1 filter paper and dilute according to the dilution table. *Typically, a dilution of 1:5 and sample volume of 0.2 mL are satisfactory.*

(c) Determination of L-glutamic acid in fruit and vegetable products.

Cut the vegetable or fruit, e.g. a tomato, into approx. 2 cm cubes and homogenise using a kitchen blender. Accurately weigh approx. 1 g of representative material into a 100 mL volumetric flask, add 50 mL of distilled water and allow to extract for 10 min. Adjust the volume to the mark with distilled water, mix and filter through Whatman No. 1 filter paper. *Typically, no dilution will be necessary and volumes up to 0.5 mL will be required.*

(d) Determination of L-glutamic acid in tomato juice.

Stir the tomato juice thoroughly, accurately weigh approx. 1 g into a 100 mL volumetric flask, adjust the volume to the mark with distilled water and mix. Filter the turbid solution through Whatman No. 1 filter paper and dilute according to the dilution table. *Typically, a dilution of 1:100 and sample volume of 0.2 mL are satisfactory.*

REFERENCE:

Beutler, H. O. (1990). L-Glutamate, Colorimetric Method with Glutamate Dehydrogenase and Diaphorase. In "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VIII**, pp. 369-376, VCH Publishers (UK) Ltd., Cambridge, UK.

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