L-Glutamic acid

Colorimetric-method
for the determination of L-glutamic acid in foodstuffs and other materials

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

For *in vitro* use only

Store at 2-8°C

Cat. No. 10 139 092 035

Test-Combination for 3 × 13 determinations

For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

L-Glutamic acid (L-glutamate) is oxidatively deaminated by nicotinamide-adenine dinucleotide (NAD) to 2-oxoglutarate in the presence of the enzyme glutamate dehydrogenase (GIDH) (1).

(1) L-Glutamate + NAD^+ + H_2O $\overset{\text{GIDH}}{\rightleftharpoons}$ 2-oxoglutarate + NADH + NH_4^+

In the reaction catalyzed by diaphorase the NADH formed converts iodonitrotetrazolium chloride (INT) to a formazan which is measured at its maximum in the visible range at 492 nm (2).

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

The equilibrium of reaction (1) lies on the side of L-glutamate. By trapping the formed NADH with INT (2), the equilibrium is displaced in favour of 2-oxoglutarate.

The Test-Combination contains

1. Bottle 1 with approx. 25 ml solution, consisting of:
   potassium phosphate/triethanolamine buffer, pH approx. 8.6; Triton X-100 (trademark of Rohm & Haas, Philadelphia, USA)
2. Three bottles 2 with each approx. 35 mg of lyophilizate, consisting of:
   diaphorase, approx. 4 U; NAD, approx. 28 mg
3. Bottle 3 with iodonitrotetrazolium chloride solution, approx. 2.5 ml
4. Bottle 4 with approx. 1.2 ml glutamate dehydrogenase solution, approx. 1080 U
5. Bottle 5 with L-glutamic acid assay control solution for assay control purposes (measurement of the assay control solution is not necessary for calculating the results.) Use the assay control solution undiluted. (Expiry date: see pack label)

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve contents of one bottle 2 with 2.5 ml redist. water.
3. Dilute contents of bottle 3 with 6 ml redist. water.
4. Use suspension 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.
The contents of the bottles 2 are stable at 2-8°C (see pack label).
Solution 2 is stable for 1 week at 2-8°C.
Bring solution 2 to 20-25°C before use.
The contents of bottle 3 are stable at 2-8°C (see pack label).
Solution 3 is stable for 3 months at 2-8°C and for 1 month at 20-25°C stored in the dark.
Bring solution 3 to 20-25°C before use.
The contents of bottle 4 are stable at 2-8°C (see pack label). A weak opalescence or a slight turbidity, respectively, is without any influence on the activity of the enzyme.

Procedure

Wavelength: (Hg) 492 nm
Glass cuvette[1]: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.030 ml
Read against air (without a cuvette in the light path), against water or blank
Sample solution: 0.4-14 µg L-glutamate/assay[2] (in 0.200-2.000 ml sample volume)

Pipette into cuvettes	Blank	Sample
solution 1[3]	0.600 ml	0.600 ml
solution 2[3]	0.200 ml	0.200 ml
solution 3[3,4]	0.200 ml	0.200 ml
sample, resp. assay control solution*	-	0.200 ml
redist. water	2.000 ml	1.800 ml
Mix**, after 2 min read absorbances of the solutions (A_1). Repeat the measurement after 2 min. If a change in absorbance greater than 0.010 is observed, the sample must be pre-treated according to pt. 7.3 (removal of reducing substances). However, if the change in absorbance is less than 0.010, such pre-treatment is not necessary, providing the reaction is started immediately after the previous measurement by addition of:		
solution 4	0.030 ml	0.030 ml
Mix**, wait until reaction has stopped (approx. 15 min), and read absorbances of the blank and sample immediately one after another (A_2). Repeat reading of absorbances after 2 min. If the reaction has not stopped, continue to read the absorbances at 2 min intervals until the absorbance increases constantly over 2 min.		

* 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)

If the absorbance A_2 increases constantly, extrapolate the absorbances to the time of addition of solution 4 (GIDH). (If the increase in absorbance is the same for blank and sample, an extrapolation is not necessary when the absorbances of blank and sample measured immediately one after another are used for the calculation of the absorbance differences.)

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

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

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

Calculation with extinction coefficient[5]

According to the general equation for calculatig the concentration:

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

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 formazan at 492 nm
= 19.9 [l × $mmol^{-1}$ × cm^{-1}].

It follows for L-glutamic acid:

$$c = \frac{3.030 \times 147.13}{19.9 \times 1.00 \times 0.200 \times 1000} \times \Delta A = 0.1120 \times \Delta A$$

[g glutamic acid/l sample solution]

If the sample has ben diluted during praparation, the result must be multiplied by the dilution factor F.

1 If desired, disposable cuvettes may be used instead of glass cuvettes.
2 See instructions for performance of assay
3 For series analysis, a suitable stock solution may be prepared by mixing solutions 1, 2 and 3. This stock solution is stable for 1 h when stored in the dark at 20°C. Use 1.000 ml for the assay.
4 INT is sensitive to light. After addition of solution 3, do not store the cuvettes in the light.
5 If results calculated with the extinction coefficient are systematically too low (and gross errors in analysis can be excluded), the calculation of results with a standard is recommended.

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Calculation with a standard[5]

According to the general equation for calculating the concentration relative to a standard

$$c = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times c_{standard} \text{ [g/l sample solution]}$$

the concentration of the sample solution is calculated from the concentration of the standard solution:

$$c = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times c_{standard} \text{ [g L-glutamic acid/l sample solution]}$$

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

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

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

1. Instructions for performance of assay

The amount of L-glutamic acid present in the assay has to be between 0.4 µg and 14 µg. In order to get a sufficient absorbance difference, the sample solution is diluted to yield an L-glutamic acid concentration of maximum 0.07 g/l.

Dilution table

Estimated amount of L-glutamic acid per liter	Dilution with water	Dilution factor F
< 0.07 g	-	1
0.07-0.7 g	1 + 9	10
0.7-7 g	1 + 99	100
> 7 g	1 + 999	1000

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 so as 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 For series analysis, a suitable stock solution may be prepared by mixing solution 1, 2 and 3. This stock solution is stable for 1 h when stored in the dark. Use 1.000 ml for each assay.

2.2 The reaction system is sensitive to light (daylight or artificial light) after the addition of INT (solution 3 or reaction mixture). The incubation has to be done in the dark:

a) if incubating in the photometer, close cuvette compartment of the photometer and block out light.

b) Cover the cuvettes or store in a darkend cupboard.

2.3 In carrying out the calculation, usually the results are to be given as L-glutamic acid (molar mass 147.13 g/mol). When calculating the results as monosodium L-glutamate monohydrate (MSG), the molar mass 187.13 g/mol has to be used. (In enzymatic determinations, the L-glutamate ion is measured.)

3. Specificity (Ref. 1)

The method is specific for L-glutamic acid.

In the analysis of commercial L-glutamic acid, results of approx. 99% have to be expected.

4. Sensitivity and detection limit (Ref. 1.2)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume v = 2.000 ml a L-glutamic acid concentration of 0.06 mg/l sample solution (if v = 0.100 ml, this corresponds to 0.3 mg/l sample solution).

The detection limit of 0.2 mg/l is derived from the absorbance difference of 0.020 and a maximum sample volume v = 2.000 ml.

5. Linearity

Linearity of the determination exists from 0.4 µg L-glutamic acid/assay (0.2 mg L-glutamic acid/l sample solution; sample volume v = 2.000 ml) to 14 µg L-glutamic acid/assay (0.07 g L-glutamic acid/l sample solution; sample volume v = 0.200 ml).

6. Precision

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 = 0.200 ml this corresponds to a L-glutamic acid concentration of approx. 0.5-1 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 0.005-0.01 g/100 g can be expected.)

The following data have been published in the literature:

CV = 0.8 %	L-glutamic acid solution		(Ref. 1.1)
CV = 0.81 %	L-glutamic acid solution	(n = 15)	
CV = 0.88 %	L-glutamic acid solution	(n = 15)	(Ref. 1.2)

Boiled finely minced pork sausage:

x = 0.13 g/100 g	r = 0.01	g/100 g	$s_{(r)} = \pm$ 0.0035 g/100 g
	R = 0.013	g/100 g	$s_{(R)} = \pm$ 0.0047 g/100 g
Tomato pulp:	r = 0.08	g/100 g	$s_{(r)} = \pm$ 0.03 g/100 g
	R = 0.11	g/100 g	$s_{(R)} = \pm$ 0.04 g/100 g

(Ref. 2.3)

7. Interference/sources of error

7.1 If the NH_4^+content of a given sample is higher than the concentration of L-glutamate, the reaction proceeds **less rapidly**.

7.2 Test-Combinations which are used beyond the stated date of expiration, or reagent mixtures of diaphorase, NAD, and INT stored for more than one hour at 20-25°C (see[3]) before being used cause a delayed reaction or produce a *creep reaction* which must be taken into account in the calculation by extrapolating of A_2 to the time of addition of solution 4 (GlDH).

7.3 High concentrations of reducing substances, e.g. L-ascorbic acid in meat additives or sulfurous acid in fruit juices interfere with the assay because of their reaction with INT and cause a *creep reaction*. This interference will be eliminated by preceding treatment of the sample with H_2O_2:

Weigh the sample or pipette the sample solution, respectively, diluted, if necessary, into a 50 ml volumetric flask. The amount of L- glutamic acid in the sample should be approx. 2 mg. Fill up with water to approx. 40 ml, add 0.5 ml KOH (2 M) and 0.01 ml H_2O_2 (30%, w/v), incubate for 10 min at approx. 70°C. Adjust to pH 7-8 with H_2SO_4 (1 M). Allow the solution to cool to 20-25°C, fill up to the mark with water, mix and filter, if necessary.

8. Recognizing interference during the assay procedure

8.1 If the conversion of L-glutamic acid 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 L-glutamic acid (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 glutamic acid contain hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulations 67/548 and 99/45 and subsequent alteration, supplementation and adaptation guidelines. Please refer to the safety date sheet or the labels of the affected vials for further information.

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 sample** to approx. pH 8 by adding sodium or potassium hydroxide solution;
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;
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.

11. Application examples

Determination of L-glutamic acid in soy sauce and condiment
Dilute the sample acc. to the dilution table. Use the diluted sample for the determination.

Determination of L-glutamic acid in meat extracts, soup or bouillon cubes
Accurately weigh approx. 1 g sample and dissolve with approx. 70 ml of water, heat at approx. 70°C for 10 min, and allow to cool to 20-25°C. Transfer into a 100 ml volumetric flask and fill up to the mark with water. Shake and filter through a filter paper moistened with the solution in order to separate the fat. In the case of residual turbidity, filter again through the same filter paper. Use the clear solution diluted, if necessary (see dilution table), for the assay.

Determination of L-glutamic acid in meat products (sausages)
Accurately weigh approx. 10 g minced sausage and homogenize with 80 ml perchloric acid (1 M) for 10 min using a homogenizer (Ultra Turrax, or IKA mill), centrifuge, decant the supernatant, and filter. Discard the first few ml of the filtrate and pipette 20 ml into a beaker, adjust to pH 10.0 with potassium hydroxide (2 M); measure the volume of KOH. To obtain quantitative precipitation of the potassium perchlorate formed, place in an ice-bath or refrigerator for 20 min; filter. Use the clear solution diluted, if necessary (see dilution table), for the assay. When calculating the dilution, take into account the water content of the sample.

For calculating the content (in g/100 g) according to the above-mentioned formula (see calculation) the content of the sample in the sample solution is needed. When applying the above-mentioned sample preparation and considering the water content of the sample the weight of the sample is calculated according to the following furmula:

$$\text{Weight}_{\text{sample}} = \frac{a \times 1000 \times d}{(b + a \times w) \times (d + e)} \quad [g/l]$$

It is:

a:	the weighed sample in g
b:	volume of perchloric acid in ml
d:	volume of filtrate for neutralization in ml
e:	volume of KOH for neutralization in ml
w:	water content of the sample in (%;w/w)/100
1000:	factor for g expressed in mg

(The specific gravity of water from the sample at 20-25°C is approx. 1 g/ml. It can be neglected for the calculation.)

Determination of L-glutamic acid in vegetable and fruit products
Accurately weigh out approx. 1 g of the homogenized sample, extract with approx. 50 ml of water (for 10 min) and transfer to a 100 ml volumetric flask, fill up to the mark with water, shake and filter. Use the clear solution diluted, if necessary, (see dilution table) for the assay.

The sample generally need not be decolorized. In the case decolorization is required, add 1% polyamide powder or polyvinylpolypyrrolidone (PVPP) to the sample solution; stir briefly (1 min) and filter.

12. Further applications

The method may also be used in the examination of pharmaceuticals, and in research when analyzing biological samples. For details of sampling, treatment and stability of the sample see Ref. 1.1, 1.2.

Determination of L-glutamic acid 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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L-Glutamic acid assay control solution (Bottle 4)

Concentration: see bottle label

L-Glutamic acid assay control solution is a stabilized aqueous solution of L-glutamic acid. It serves as an assay control solution for the enzymatic analysis of L-glutamic acid in foodstuffs and other materials.

Application:

1. Addition of L-glutamic acid assay control solution to the assay mixture:
Instead of sample solution the assay control solution is used for the assay.

2. Restart of the reaction, quantitatively:
After completion of the reaction with sample solution and measuring of A_2, add 0.100 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 15 min.) Calculate the concentration from the difference of (A_3-A_2) 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 data stated on the bottle label.

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
solution 1	0.600 ml	0.600 ml	0.600 ml	0.600 ml
solution 2	0.200 ml	0.200 ml	0.200 ml	0.200 ml
solution 3	0.200 ml	0.200 ml	0.200 ml	0.200 ml
sample solution	-	0.200 ml	-	0.100 ml
assay control sln.	-	-	0.200 ml	0.100 ml
redist. water	2.000 ml	1.800 ml	1.800 ml	1.800 ml
Mix, and read absorbances of the solutions (A_1) after approx. 2 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:

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

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