Tentative Translation

JAS 0032

JAPANESE AGRICULTURAL STANDARD

Determination of the 4-aminobutanoic acid (GABA) in rice

— High performance liquid chromatographic method

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Ministry of Agriculture, Forestry and Fisheries

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Food and Agricultural Materials Inspection Center, Incorporated Administrative Agency

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Foreword

This Japanese Agricultural Standard has been established by the Minister of Agriculture, Forestry and Fisheries through deliberations at the Council for the Japanese Agricultural Standards as a result of proposal for the establishment of Japanese Agricultural Standard submitted by Food and Agricultural Materials Inspection Center, Incorporated Administrative Agency with the original bill being attached, based on the provisions of Article 4, paragraph (1) of the Act on Japanese Agricultural Standards.

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JAPANESE AGRICULTURAL STANDARD JAS
(Tentative Translation) 0032 : 2025

Determination of the 4-aminobutanoic acid (GABA) in rice

— High performance liquid chromatographic method

WARNING — The user of this document should be familiar with normal laboratory practice. This document does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this document to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This document specifies a high performance liquid chromatographic method for the determination of 4-aminobutanoic acid (GABA) (also called γ-aminobutyric acid) in the rice [limited to husked rice (rice prepared by removing the husk from paddy), milled rice (rice with all or some of the bran layer and germ removed from husked rice), and husked rice that has been allowed to germinate and then stopped growing, including its milled form, the same applies hereinafter].

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. The latest edition of the referenced document (including any amendments) applies.

ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

JIS K 0557, *Water used for industrial water and wastewater analysis*

JIS K 8576, *Sodium hydroxide (Reagent)*

JIS K 8589, *5-Sulfosalicylic acid dihydrate (Reagent)*

3 Terms and definitions

No terms and definitions are listed in this document.

4 Principle

GABA is extracted with sulfosalicylic acid solution from the ground test sample. Sodium hydroxide solution is added to the sample extract to adjust the pH, and then make up to volume with dilute hydrochloric acid. The GABA in the sample extract is determined by high performance liquid chromatograph (HPLC) system capable of post-column derivatization.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

WARNING — It is the responsibility of users of this document to comply with legal regulations regarding the use of reagents.

5.1 Water, conforming to grade A3 or A4 of JIS K 0557.

5.2 Standard reagent, either of the following:

a) GABA, of minimum mass fraction, ≥ 98,0%;

b) commercially available amino acid standard solution prepared with GABA at a concentration suitable for HPLC system and operating conditions.

5.3 Hydrochloric acid, of mass fraction, 35,0 % to 37,0 %, of suitable quality for post-column derivatization.

NOTE In the interlaboratory test described in Annex A, when ninhydrin was used as the derivatization reagent, hydrochloric acid with ninhydrin-positive substances removed was used, and when *o*-phthalaldehyde was used as the derivatization reagent, hydrochloric acid of 35,0 % to 37,0 %, according to JIS K 8180 was used.

5.4 Sodium hydroxide, of minimum mass fraction, 97,0 %, according to JIS K 8576.

5.5 5-Sulfosalicylic acid dihydrate, of minimum mass fraction, 99,0 %, according to JIS K 8589.

5.6 Dilute hydrochloric acid, prepared by adding approximately 0,86 ml of hydrochloric acid to water and making up to a final volume of 1,0 l.

5.7 Sodium hydroxide solution, dissolve 12 g of sodium hydroxide per 100 ml of water.

WARNING — Since irritating mist is generated, work shall be done in a place with good ventilation inside a fume cupboard etc.

5.8 Sulfosalicylic acid solution, prepared by dissolving 116,5 g of 5-Sulfosalicylic acid dihydrate in water and making up to a final volume of 1,0 l.

5.9 Reagents for HPLC analysis, necessary for HPLC system and operating conditions, with suitable quality.

5.10 A series of standard solutions, made by dissolving the standard reagent in dilute hydrochloric acid and preparing it to a concentration of 4 or more stepwise concentrations suitable for HPLC system and the operating conditions to be used. The minimum concentration of the standard solution shall be set at or above the minimum limit of quantitation of HPLC system (see 8.2.1 a)).

NOTE In the interlaboratory test described in Annex A, a series of standard solutions was prepared with a GABA concentration of 10 mg/kg to 500 mg/kg for the test samples.

6 Apparatus

The usual laboratory apparatus and the following shall be used.

6.1 Electronic analytical balances, capable of weighing to an accuracy of ±0,1 mg.

6.2 Extraction container, made of glass or resin that is resistant to acidic solutions, with the lid, of approximately 50 ml capacity, capable of maintaining necessary space for enough shaking.

6.3 Shaker, capable of mixing an extraction container, by shaking vertically, at 100 r/min or more.

6.4 One-mark volumetric flasks, capacity suitable for either the preparation of standard solutions (see 5.10) or extraction (see 8.1), or both procedures, of ISO1042, class A.

6.5 Centrifuge tubes, made of resin that is resistant to acidic solutions, with the lid. They shall be able to withstand centrifugation set by the centrifuge.

6.6 Centrifuge, capable of 2 000×*g* or more.

WARNING— To prevent accidents, operate the centrifuge in accordance with the instruction manual of the equipment.

6.7 Membrane filters, made of hydrophilic polytetrafluoroethylene (PTFE), suitable for acidic solutions, with a pore size of 0,45 µm or less. The filter and the housing shall be unitary, and the housing material shall be resistant to acidic solutions.

NOTE In the interlaboratory test described in Annex A, 25 mm diameter membrane filters connected to luer lock

syringes were used.

6.8 HPLC, capable of post-column derivatization, equipped with either a fluorescence detector or a visible absorption detector on the detection part. They shall be able to set the operating conditions as specified in 8.2.1.

7 Preparation of test samples

Grind the sample to homogeneity using a mechanical grinder or the like to obtain the test sample. Immediately perform the procedure in 8.1, or freeze the test sample to store. When storing the test sample frozen, transfer all of them, or a portion of them stirred until homogeneous, into a sealable container soon after ground. Return it to room temperature and mix well before use.

NOTE It has been confirmed that the test samples remain stable for at least 8 weeks when stored frozen at –15 °C or below.

8 Procedure

8.1 Extraction

8.1.1 Weigh, to the nearest 10 mg, approximately 2 g of the test sample into an extraction container, add 25 ml of sulfosalicylic acid solution and shake immediately to suspend the entire test sample.

8.1.2 Shake for 20 minutes by shaker, then add 5 ml of sodium hydroxide solution and shake to mix.

8.1.3 Transfer the mixture in the extraction container (see 8.1.2) to a 50 ml one-mark volumetric flask. Add dilute hydrochloric acid to the residue in the extraction container, and transfer the entire residue to the flask. Make up to volume with dilute hydrochloric acid and mix it. Use this as the mixed solution.

8.1.4 Transfer some or all of the mixed solution (see 8.1.3) to a centrifuge tube, centrifuge it for 10 minutes at 2 000 ×*g* or more.

8.1.5 Filter some of the supernatant liquid through a membrane filter (discard the first filtrate), and use as the sample extract. Perform the operation in 8.2 continuously on the same day or store the sample extract at –15 °C or below. If it is stored at –15 °C or below, return it to room temperature and mix using a test tube mixer, etc. to fully dissolve the undissolved matter before use.

NOTE1 In the interlaboratory test described in Annex A, approximately 0,5 ml of the first filtrate was discarded.

NOTE2 It has been confirmed that the pH of the sample extract is approximately 2,2.

NOTE3 It has been confirmed that the sample extract remains stable for at least 17 weeks when stored at –15 °C or below.

8.2 Determination

8.2.1 Setting of operating conditions for HPLC system and confirmation of its performance

Operate system in accordance with the instruction manual, and set the operating conditions to meet the following criteria:

a) The minimum limit of quantitation shall be 10 mg/kg or less as test sample concentration.

b) The GABA peak shall be separated from the peaks that precede and succeed it, so as not to interfere with the measurement of the standard solution and the sample extract.

c) The coefficient of determination of the linear calibration curve shall be 0,990 or more.

8.2.2 Measurement with HPLC system

Confirm that the fluctuation of base line gives no hindrance for determination of GABA by a blank run under

the condition of above-mentioned setting, and inject a series of standard solutions and the same amount of the sample extract (see 8.1.5) into HPLC system. Confirm the fluctuation of sensitivity during measurement.

NOTE In the interlaboratory test described in Annex A, an intermediate concentration solution of the series of standard solutions was measured every 6 measurement of the sample extracts, and it was confirmed that the fluctuation was within ±10 %.

9 Calculation

9.1 General

Quantitative determination is performed by the external standard method with integration of the peak area, which is then related to the corresponding value for the standard substance.

9.2 Quantitation

Perform a linear regression of peak areas for each of the series of standard solution against the GABA concentrations of the respective standard solutions to create a calibration curve.

Calculate the concentration of GABA from the area of sample extract by the calibration curve. The GABA content in the test sample, w_i, is given by the formula:

$$w_i = \frac{C \times V}{W}$$

where

w_i is the GABA content in the test sample (mg/kg);

C is the concentration of GABA in the sample extract (μg/ml);

V is the final volume (ml) (see 8.1.3);

W is the mass(g) of the test sample(see 8.1.1).

9.3 Expression of results

Express the results to two significant figures.

10 Precision

10.1 Interlaboratory test

An interlaboratory test was carried out to determine the precision of the test method, and the results are summarized in Annex A. The values derived from this interlaboratory test can be inapplicable to the content ranges other than the given one (2,3×10 mg/kg to 3,6×10^2 mg/kg) nor the matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, is expected in not more than 5 % of cases to be greater than the repeatability limit (r) values [2] given in Table A.1 as long as the specified operation is correctly done [3].

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, is expected in not more than 5 % of cases to be greater than the reproducibility limit (R) values [2] given in Table A.1 as long as the specified operation is correctly done [3].

11 Quality control

The laboratory shall have internal quality control procedures for tests.

12 Test report

The test report shall include at least the following information:

a) the title or the reference number of this document;

b) every detail to identify the test sample;

c) the date of the test;

d) the results of the test.

Annex A
(informative)

Results of interlaboratory test

An interlaboratory test was carried out in accordance with IUPAC protocol [4] in 2023 in Japan, and gave the statistical results given in Table A.1. Commercially available rice (husked rice, milled rice, germinated husked rice) were ground at 10 000 r/min by mechanical grinder.

After the homogeneity [5] was confirmed, each of the ground material was used as a test sample. The experimental protocol, the GABA standard reagent and test samples were supplied to the participating laboratories by the Food and Agricultural Materials Inspection Center (FAMIC), the organizer of this interlaboratory test. Each laboratory tested a total of 12 test samples (6 pairs of blind duplicates) according to the experimental protocol.

Table A.1 — Precision data

Sample identification	**Sample 1 (milled rice)**	**Sample 2 (milled rice)**	**Sample 3 (husked rice)**	**Sample 4 (germinated husked rice)**	**Sample 5 (germinated husked rice)**	**Sample 6 (germinated husked rice)**
Number of participating laboratories	10	10	10	10	10	10
Number of accepted test results	9	10	10	8	9	9
Mean GABA content, mg/kg (mass fraction)	22,8	35,0	56,4	58,3	148,3	359,7
Repeatability standard deviation s_r mg/kg	1,0	0,61	1,0	0,93	2,0	5,0
Repeatability relative standard deviation, RSD_r, %	4,3	1,7	1,8	1,6	1,3	1,4
Repeatability limit r (r = 2,8 s_r) mg/kg	2,7	1,7	2,8	2,6	5,5	14
Reproducibility standard deviation s_R mg/kg	1,6	1,6	2,3	1,9	3,8	8,1
Reproducibility relative standard deviation, RSD_R, %	6,8	4,7	4,0	3,3	2,5	2,2
Reproducibility limit R (R = 2,8 s_R) mg/kg	4,4	4,6	6,4	5,3	11	23

NOTE1 Considering the usage situation in Japan, the HPLC system capable of post-column derivatization and equipped with either a fluorescence detector or a visible absorption detector on the detection part was chosen for the interlaboratory test. The details of the HPLC system used were as follows: the fluorescence detectors were used in 4 laboratories and the visible absorption detectors were used in 6 laboratories.

NOTE2 Examples of the operating conditions adopted in the interlaboratory test are shown below. This information is given for the convenience of users of this document and does not constitute an endorsement of the products.

EXAMPLE 1 Detector: fluorescence detector;

Column: AApak Na-LG (50 mm×6,0 mm i.d., JASCO);

Mobile phase: AminoBuffer Reagent Na-LG set;

Flow rate: 0,5 ml/min;

Injection volume: 5 µl;

Column temperature: 60 °C;

Derivatization reagent: *o*-phthalaldehyde;

Excitation wavelength: 345 nm;

Emission wavelength: 455 nm;

Run time: 60 min.

EXAMPLE 2 Detector: visible absorption detector;

Column: #2622PH (60 mm×4,0 mm i.d., Hitachi);

Mobile phase: Buffer set for protein hydrolysate PH-SET;

Flow rate: 0,4 ml/min;

Injection volume: 20 µl;

Column temperature: 57 °C;

Derivatization reagent: ninhydrin;

Wavelength: 570 nm;

Run time: 53 min.

Bibliography

[1] JIS K 8180, *Hydrochloric acid (Reagent)*

[2] ISO 5725-6:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 6: Use in practice of accuracy values*

NOTE Section 4 "Determination of limits" of the referenced document was referred to for the calculation of the repeatability limit and the reproducibility limit.

[3] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

NOTE Section 7.1.5 of the referenced document was referred to for the expression of the repeatability limit and the reproducibility limit.

[4] Horwitz, W., Protocol for the design, conduct and interpretation of method-performance studies, Pure & Appl. Chem., 1995, **67**(2), pp. 331–343.

[5] Thompson, M., et al., The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories, *Pure & Appl. Chem.*, 2006, **78**(1), pp. 145-196.

NOTE Section 3.11 "Testing for sufficient homogeneity and stability" of the referenced document was referred to for the method to confirm the homogeneity.