Tentative Translation

JAS 0016

JAPANESE AGRICULTURAL STANDARD

Determination of the ornithine in mushroom (*Hypsizygus marmoreus*) — High-performance liquid chromatographic method

Date of Establishment: 2021 – 3 – 31

Ministry of Agriculture, Forestry and Fisheries

Precautions for using English version of JAS

This English translation has been made by the drafting party etc., based on the original text (Japanese version), and has been posted on the website of Food and Agricultural Materials Inspection Center (FAMIC), Incorporated Administrative Agency, with permission of the publisher of the original text (Ministry of Agriculture, Forestry and Fisheries).
The translation is made in consideration of technical contents, but it is aimed to provide information when using JAS original text, and is not recognized as having the same effects as the original text.
If there is any doubt in the translation, please follow the original.
FAMIC is not responsible for inconvenience by using only the translation.

Food and Agricultural Materials Inspection Center, Incorporated Administrative Agency

Contents

Page

1 Scope 1

2 Normative references 1

3 Terms and definitions 1

4 Principle 1

5 Reagents 1

6 Apparatus 2

7 Preparation of test samples 3

8 Procedure 3

8.1 Extraction 3

8.2 Determination 3

9 Calculation 4

9.1 Quantitation 4

9.2 Expression of results 5

10 Precision 5

10.1 Interlaboratory test 5

10.2 Repeatability 5

10.3 Reproducibility 5

11 Quality control 5

12 Test report 5

Annex A (informative) Results of interlaboratory tests 6

Bibliography 8

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, based on the provision of Article 4, paragraph (1) of the Act on Japanese Agricultural Standards as applied mutatis mutandis pursuant to the provision of Article 5 of the Act.

Attention is drawn to the possibility that some parts of this Standard may conflict with patent rights, applications for a patent after opening to the public or utility model rights. The Minister of Agriculture, Forestry and Fisheries and the Council for the Japanese Agricultural Standards are not responsible for identifying any of such patent rights, applications for a patent after opening to the public or utility model rights.

JAPANESE AGRICULTURAL STANDARD JAS
(Tentative Translation) 0016 : 2021

Determination of the ornithine in mushroom (*Hypsizygus marmoreus*)

— 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 test method for the determination of ornithine in mushroom (*Hypsizygus marmoreus*) (fresh).

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 editions of the referenced documents (including any amendments) apply.

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

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

JIS K 8180, *Hydrochloric acid (Reagent)*

3 Terms and definitions

No terms and definitions are listed in this document.

4 Principle

Ornithine is extracted from a test portion of finely chopped mushroom with dilute hydrochloric acid. The ornithine in the extract is determined with high-performance liquid chromatograph (HPLC) system that meets the specified criteria.

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) Ornithine monohydrochloride, of minimum mass fraction, ≥ 97,0%; or

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

5.3 Hydrochloric acid, of minimum mass fraction, 35,0 %, according to JIS K 8180.

WARNING — Since it is a deleterious substance, avoid contact with eyes, mucous membranes and skin.

5.4 Dilute hydrochloric acid, prepared by adding 0,86 mL of hydrochloric acid to water and obtaining a constant volume of 1,0 L.

5.5 Internal standard, necessary when using the internal standard method for the measurement (see 8.2), and confirmed to be neither included in *Hypsizygus marmoreus*, nor overlapping other ingredient peaks therein. Suitable for HPLC system and operating conditions.

NOTE Norvaline and theanine were used on the HPLC system equipped with a tandem mass spectrometer in the interlaboratory tests described in Annex A.

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

5.7 A series of standard solutions,

made by dissolving the standard reagent in dilute hydrochloric acid and preparing it to a concentration of 3 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 tests described in Annex A, a series of standard solutions was prepared with an ornithine concentration of 400 mg/kg to 2 500 mg/kg for the test samples.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Electronic analytical balance, capable of weighing to a readability of ±0,1 mg, capable of weighing more than 200 g.

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 shaking an extraction container, back and forth, at 100 r/min or more.

6.4 One-mark volumetric flasks, capacity suitable for the preparation of standard solutions (see 5.7) and extraction procedures (see 8.1.2 and 8.1.3), of ISO 1042, 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 at 13 000 ×*g*.

6.6 Centrifuge, capable of carrying out centrifugation at 13 000 ×*g* with centrifuge tubes.

WARNING — To prevent accidents, operate the centrifuge according to the instruction manual of the equipment.

6.7 Membrane filters, made of hydrophilic polytetrafluoroethylene (PTFE), suitable for acidic solutions, with a nominal pore size of 0,2 µm. The filter and the housing shall be unitary, and the material of the housing shall be resistant to acidic solutions.

6.8 Vials, suitable for HPLC system to be used, made of deactivated glass or high purity polypropylene. The septum of the lid shall be made of or coated with PTFE.

6.9 HPLC system,

equipped with HPLC on the separation part, and either a fluorescence detector, a visible absorption detector (both detectors shall carry out the measurement by the post-column derivatization method), or a tandem mass spectrometer on the detection part. They shall be able to set the operating conditions as specified in 8.2.1.

7 Preparation of test samples

After removing the hard tip of the sample, finely chop it to make it visually homogeneous with a food processor or the like. Use this as a test sample. Immediately carry out the extraction specified in 8.1, or keep the test sample frozen. To keep it frozen, transfer the whole test sample, or a portion thereof which is stirred to be homogeneous, into the container that is capable of being tightly sealed, as soon as the test sample is prepared. Take the test sample out of the freezer before use, allow it to return to room temperature and mix well.

NOTE It has been confirmed that the test sample will remain stable for at least 20 weeks when kept frozen at -30 °C to -20 °C.

8 Procedure

8.1 Extraction

8.1.1 Weigh, to the nearest 10 mg, approximately 2 g of the test sample (see Clause 7) into an extraction container, add 15 mL of dilute hydrochloric acid and shake it with a shaker for 15 min.

8.1.2 Transfer the mixture in the extraction container (see 8.1.1) to a one-mark volumetric flask. Add dilute hydrochloric acid to the residue in the extraction container, and transfer the entire residue to the flask.

NOTE 50 mL one-mark volumetric flasks were used in the interlaboratory tests described in Annex A.

8.1.3 Fill the flask (see 8.1.2) up to the mark with dilute hydrochloric acid and mix it. Use this as a mixed solution. When adopting the internal standard method on the measurement (see 8.2), add the internal standard before filling the flask or when diluting the measurement solution (see 8.1.5) in 8.2.2.

8.1.4 Transfer some or all of the mixed solution (see 8.1.3) to a centrifuge tube, centrifuge it for 10 min at 13 000 ×*g*.

8.1.5 Filter supernatant liquid through a membrane filter. Use this as a measurement solution. Perform the measurement (see 8.2) on the day of preparation or keep it frozen.

NOTE It has been confirmed that the measurement solution will remain stable for at least 18 weeks when kept frozen at -30 °C to -20 °C.

Take the measurement solution stored at -30 °C to -20 °C out of the freezer before use and mix it well.

8.2 Determination

8.2.1 Setting of operating conditions for HPLC system and confirmation of extraction conditions

The measurement shall be performed by the absolute calibration curve method or the internal standard method. Operate HPLC system according to the instruction manual, and set the operating conditions to meet the following criteria:

a) The minimum limit of quantitation shall be 50 mg/kg or less as test sample (see Clause 7) concentration;

b) The ornithine 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 measurement solution (or its dilution);

c) The correlation coefficient of the linear calibration curve shall be 0,995 or more;

d) Perform a recovery test by adding approximately the same amount of ornithine[1] as contained in a random test sample (see Clause 7) to the sample, and the recovery shall be within the range shown in Table 1[2];

Table 1— Recovery range

Added concentration	Recovery （%）
≧1 000 mg/kg	95 to 105
≧100 mg/kg	90 to 107
≧10 mg/kg	80 to 110

e) Determine random test samples (see Clause 7) 6 times or more[3] under repeatability condition, and the standard deviation (mass fraction) (*Srl*) shall meet the following condition[4]:

$$\frac{S_{rl}}{0{,}02\,w^{\ 0{,}85}} \leqq 1{,}3$$

where

w is the concentration of ornithine in the test sample (mass fraction)

8.2.2 Measurement with HPLC system

Confirm that the fluctuation of base line gives no hindrance for determination of ornithine by a blank run under the condition of above-mentioned setting, and inject a series of standard solutions and the same amount of the measurement solution (see 8.1.5) into HPLC system. Confirm the fluctuation of sensitivity during measurement.

NOTE In the interlaboratory tests described in Annex A, an intermediate concentration solution of the series of standard solutions was measured at 1 intervals for 5 measurement solutions, and it was confirmed that the fluctuation was within ±10 %.

Obtain the output signal of ornithine for each injected standard solution and injected measurement solution. Prior to measurement, the measurement solution may be diluted to an ornithine concentration suitable for the HPLC system.

9 Calculation

9.1 Quantitation

Construct linear calibration curve for each standard solution from the ornithine concentrations against the output signal intensity. Confirm that the following conditions are met:

a) The correlation coefficient of the linear calibration curve shall be 0,995 or more;

b) The ornithine concentration of the measurement solution (or its dilution) shall be within the calibration curve range.

Calculate the ornithine concentration (μg/mL) in the measurement solution from the output signal intensity of each measurement solution (or its dilution) by the calibration curve. The ornithine content in the test sample, wi (mg/kg), is given by the following formula:

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

where

C is the ornithine concentration in the measurement solution, in μg/mL;

V is the constant volume (mL) at filling up (see 8.1.3);

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

NOTE In the interlaboratory tests described in Annex A, V was 50.

9.2 Expression of results

Express the results to two significant figures.

10 Precision

10.1 Interlaboratory test

The 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 ($6{,}7\times10^2$ mg/kg to $1{,}9\times10^3$ 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[5] shown in Table A.1 on average as long as the specified operation is correctly done[6].

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[5] given in Table A.1 on average as long as the specified operation is correctly done[6].

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 of this document or the reference number;

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 tests

Interlaboratory tests were carried out in accordance with IUPAC protocol[7] in 2020 in Japan, and gave the statistical results shown in Table A.1. Commercially available *Hypsizygus marmoreus*, from which the hard tips were removed, were finely chopped for 5 min at 3 600 r/min by food processor. After the homogeneity [8] was confirmed, finely chopped samples were used as a test sample. The experimental protocol, the ornithine standard reagent and test samples were supplied to the participating laboratories by the Food and Agricultural Materials Inspection Center (FAMIC), the organizer of the interlaboratory tests. Each laboratory tested a total of 10 test samples (5 pairs of blind duplicates) according to the experimental protocol.

Table A.1 — Precision data

Sample identification	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Number of participating laboratories	10	10	10	10	10
Number of accepted test results	10	8	10	9	8
Mean ornithine content, mg/kg	671	895	1 216	1 538	1 853
Repeatability standard deviation s_r, mg/kg	12	12	44	40	57
Repeatability relative standard deviation, %	1,9	1,4	3,7	2,6	3,1
Repeatability limit r (r = 2,8 s_r), mg/kg	35	34	124	111	161
Reproducibility standard deviation s_R, mg/kg	46	31	86	59	60
Reproducibility relative standard deviation, %	6,9	3,5	7,1	3,8	3,2
Reproducibility limit R (R = 2,8 s_R), mg/kg	129	88	240	164	167

NOTE 1 Considering the usage situation in Japan, the HPLC system equipped with HPLC on the separation part, and either a fluorescence detector, a visible absorption detector or a tandem mass spectrometer on the detection part were chosen for the interlaboratory tests. The details of the HPLC system used are as follows: the fluorescence detectors (post-column derivatization method) were used in 2 laboratories, the visible absorption detectors (post-column derivatization method) were used in 3 laboratories, and the tandem mass spectrometers were used in 5 laboratories.

NOTE 2 Examples of the operating conditions adopted in the interlaboratory tests are shown below. This information is given for the convenience of users of this document and does not constitute an endorsement of the products by Ministry of Agriculture, Forestry and Fisheries, Japan.

EXAMPLE 1 Detector: tandem mass spectrometer;
Column: ACQUITY UPLC BEH Amide (150×2.1 mm i.d., Waters);
Mobile phase A: water-formic acid (99,7/0,3, v/v);
Mobile phase B: acetonitrile-water-formic acid (95/4,7/0,3, v/v);
Flow rate: 0,3 mL/min;
Injection volume: 10 µL;
Column temperature: 30 °C;
Ionization mode: ESI Positive;
Precursor ion (m/z): 133,00;
Product ions (m/z): 69,80 and 115,92;

Run time: 18 min

EXAMPLE 2 Detector: fluorescence detector (post-column derivatization method);
Column: Shim-pack Amino-Na (100×6,0 mm i.d., Shimadzu);
Mobile phase: Mobile phase kit for amino acid analysis (Na Type);
Flow rate: 0,3 mL/min to 0,4 mL/min;
Injection volume: 10 µL;
Column temperature: 60 °C;
Derivatization reagent: o-phthalaldehyde;
Excitation wavelength: 350 nm;
Emission wavelength: 450 nm;
Run time: 53 min

EXAMPLE 3 Detector: visible absorption detector (post-column derivatization method);
Column: Packed column for bioanalysis # 2622 (60×4,6 mm i.d., Hitachi);
Mobile phase: Buffer for biological fluid PF-SET;
Flow rate: 0,35 mL/min;
Injection volume: 20 µL;
Column temperature: 30 °C to 70 °C;
Derivatization reagent: ninhydrin;
Wavelength: 570 nm;
Run time: 125 min

Bibliography

[1] Official Methods of Analysis (2019) 21st Edition, AOAC INTERNATIONAL

NOTE "Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis" of the referenced document was referred to for the amount of ornithine to be added in the recovery test.

[2] Codex Alimentarius Commission Procedural Manual (2019) Twenty-seventh edition, World Health Organization, Food and Agricultural Organization of the United Nations

NOTE "Table 1: Guidelines for establishing numeric values for the criteria" in "Principles for the Establishment of Codex Methods of Analysis" of the referenced document was referred to for the recovery range.

[3] Validation of Analytical Procedures: Text and Methodology Q2(R1) (2005), ICH Harmonised Tripartite Guideline

NOTE "5.1. Repeatability b)" of the referenced document was referred to for the repeatability.

[4] Codex, Guidelines on Analytical Terminology, CAC/GL 72-2009

NOTE The formula was derived from the range of RSD (r) / PRSD (R) shown in "HorRat (Horwitz ratio)" of the referenced document.

[5] 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.

[6] 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.

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

[8] 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.

History of Enactment

Enactment	Public Notice of the Ministry of Agriculture, Forestry and Fisheries No. 445 of March 31, 2021

Enactment Provisions, Amended Provisions and Supplementary Provisions (Extract)

— Public Notice of the Ministry of Agriculture, Forestry and Fisheries No. 445 of March 31, 2021

This public notice comes into effect on April 30, 2021.