Tentative Translation

JAS 0024

JAPANESE AGRICULTURAL STANDARD

Determination of the procyanidins in apple juice —High performance liquid chromatographic method

Date of Establishment: 2022 - 3 - 31

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 the result of proposal for 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 provision of Article 4, paragraph (1) of the Act on Japanese Agricultural Standards.

Being in conformance with this standard may come under the use of the patent rights held by the following:

—Name: National Agriculture and Food Research Organization

—Address: 3-1-1 Kannondai, Tsukuba, Ibaraki, Japan

—Patent number: Japanese Patent No, 6508741

—Title of invention: Analysis method and analysis system of procyanidins

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JAPANESE AGRICULTURAL STANDARD JAS
(Tentative Translation) 0024 : 2022

Determination of the procyanidins in apple juice —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 procyanidins in apple juice (limited to not from-concentrate juice, 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 648, *Laboratory glassware — Single-volume pipettes*

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

JIS K 0124, *General rules for high performance liquid chromatography*

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

JIS K 8034, *Acetone*

JIS K 8355, *Acetic acid (Reagent)*

JIS K 9502, *L(+)-Ascorbic acid (Reagent)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
procyanidins
polymers formed from catechin or epicatechin

Note 1 to entry: Procyanidins are, for example, procyanidin B2.

4 Principle

Procyanidins are extracted from apple juice with acetone/water mixture. The procyanidins in the extract are determined with high performance liquid chromatograph (HPLC) system with a fluorescence detector. The concentration of procyanidins is calculated from peak area ratio of procyanidins in the extract to standard substance, procyanidin B2.

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 Procyanidin B2, confirmed purity.

5.3 Acetone, of minimum mass fraction, 99,5 %, according to JIS K 8034.

5.4 Acetic acid, of minimum mass fraction, 99,7 %, according to JIS K 8355.

5.5 Acetonitrile, HPLC grade.

5.6 Methanol, HPLC grade.

5.7 L(+)-ascorbic acid, of minimum mass fraction, 99,6 %, according to JIS K 9502.

5.8 L(+)-ascorbic acid-containing acetone/acetic acid/water mixture, mix 70 parts per volume of acetone with 0,5 parts per volume of acetic acid and 29,5 parts per volume of water, and dissolve 0,5 g of L(+)-ascorbic acid per 1,0 L of the mixture.

5.9 L(+)-ascorbic acid-containing acetic acid solution, dissolve 50 mL of acetic acid and 5 g of L(+)-ascorbic acid per 1,0 L of water.

5.10 HPLC mobile phase

5.10.1 Mobile phase A, acetonitrile/acetic acid mixture,

mix 98 parts per volume of acetonitrile with 2 parts per volume of acetic acid. Degas the mixture before use.

NOTE Degassing prevents troubles of bubbles, then gives stable flow and background.

5.10.2 Mobile phase B, methanol/acetic acid/water mixture,

mix 95 parts per volume of methanol with 2 parts per volume of acetic acid and 3 parts per volume of water. Degas the mixture before use.

NOTE Degassing prevents troubles of bubbles, then gives stable flow and background.

5.11 A series of standard solutions,

dissolve procyanidin B2 in L(+)-ascorbic acid-containing acetone/acetic acid/water mixture and prepare it to a concentration of 3 or more stepwise concentrations suitable for testing samples. Concentrations of standard solutions shall be corrected by purity of procyanidin B2.

NOTE1 In the interlaboratory tests described in Annex A, standard solutions were prepared to 10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL and 100 μg/mL of concentrations.

NOTE2 It has been confirmed that 100 μg/mL standard solution remains stable for at least 28 days when kept at −20 °C.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

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

6.2 One-mark volumetric flasks, capacity suitable for the extraction procedure (see 8.1), of ISO 1042, class A.

6.3 Single volume pipettes, capacity suitable for the extraction procedure (see 8.1), of ISO 648, class A.

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

6.5 HPLC system

6.5.1 HPLC, equipped with a degasser, a liquid-sending pump that can perform binary gradient elution, a sample injector with cooling function, a column compartment with thermostatically controlling function, a fluorescent detector that allowed to set a measurement wavelength described in 8.2.1 and data collection/integration system, prescribed in JIS K 0124.

6.5.2 Chromatographic column for HPLC, normal-phase columns, with the following characteristics:

—stationary phase: diol phase;

—capable to separate a procyanidins peak and a catechin or epicatechin peak enough.

NOTE In the interlaboratory tests described in Annex A, the columns were used, that were 4,6 mm internal diameter and 250 mm length stainless pipes packed with 5 μm spherical particle size silica gel bonded with dihydroxypropyl group chemically.

7 Test samples

Use ordinary temperature apple juice as test samples.

8 Procedure

8.1 Extraction

8.1.1 Measure 2 mL of the well-shaking test sample (see Clause 7) into 10 mL one-mark volumetric flask with a single volume pipette, and add 7 mL of acetone.

8.1.2 Fill the flask (see 8.1.1) up to the mark with L(+)-ascorbic acid-containing acetic acid solution and mix it. Filter the mixed solution through membrane filter, and use it as the measurement solution.

8.1.3 Perform the measurement in 8.2.1 and 8.2.2 on the day of preparation.

8.2 Measurement

8.2.1 Setting of HPLC system conditions

Set up the HPLC system conditions in accordance with the manufacturer's instructions and adjust it as follows. In this conditions, peaks of catechin or epicatechin and peaks of procyanidins shall be separated.

a) Column temperature: 30 °C to 35 °C;

b) Measurement wavelength (excitation and emission): 230 nm and 321 nm;

c) Volume injected: 1 μL to 10 μL;

d) Sample cooler temperature: 4 °C;

e) Binary gradient conditions: for 1 min to 2 min after injecting, mix and develop 91 % to 95 % mobile phase A and 5 % to 9 % mobile phase B, then increase the volume ratio of mobile phase B to 90 % to 98 % quickly in order to elute other substances from the column.

NOTE In the interlaboratory tests described in Annex A, the conditions described in Table 1 were used.

Table 1 — HPLC system conditions in the interlaboratory tests

Flow rate of the mobile phase	1,0 mL/min		
Temperature of the column	35 °C		
Temperature of the detector	40 °C		
Excitation wavelength	230 nm		
Emission wavelength	321 nm		
Volume injected	5 µL		
Temperature of the sample cooler	4 °C		
Mixing volume	0,5 mL		
Binary gradient conditions	Time (min)	Mobile phase A (%, v/v)	Mobile phase B (%, v/v)
	0 to 1,5	93	7
	1,5 to 10	2	98
	10 to 20	93	7
NOTE The values given are examples.			

8.2.2 Measurement with HPLC system

Run the entire HPLC system to stabilize it. Confirm that the fluctuation of base line gives no hindrance for determination of procyanidins by a blank run under the specified conditions (see 8.2.1). Inject a series of standard solutions and the same amount of the measurement solutions (see 8.1) 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 every 7 measurement of the measurement solutions, and it was confirmed that the fluctuation was within ±10 %.

8.2.3 Identification

Identify the individual procyanidins peak in the sample chromatogram by comparing the retention times with those of procyanidin B2 obtained from the standard solutions under the same chromatographic conditions. Include the peak immediately following procyanidin B2 peak in the procyanidins peak.

NOTE Typical HPLC chromatogram of apple juice is given in Annex B.

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

Obtain the areas of procyanidin B2 in each of a series of standard solutions. Construct linear calibration graphs for each standard of the procyanidin B2 concentrations against the peak areas obtained from the data collection/integration system. The correlation coefficient of the linear calibration is required to be 0,995 or more.

Calculate the concentration of procyanidins from the area of each measurement solution by the calibration curve. The procyanidins content in test sample, ρ, is given by the formula:

$$\rho = \frac{\rho_S \times V}{V_S}$$

where

ρ is the procyanidins content in the test sample (mg/L)

ρ_S is the concentration of procyanidins in the measurement solution (μg/mL)

V_S is the volume (mL) of the test sample (see in 8.1.1)

V is the constant volume (mL) at filling up (see in 8.1.2)

9.3 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 (8,9×10 mg/L to 2,5×10^2 mg/L) 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 [1] given in Table A.1 on average as long as the specified operation is correctly done [2].

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 [1] given in Table A.1 on average as long as the specified operation is correctly done [2].

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 tests

Interlaboratory tests were carried out in accordance with IUPAC protocol [3] in 2021 in Japan, and gave the statistical results given in Table A.1. After the homogeneity [4] was confirmed, commercially available apple juice, well shaken at ordinary temperature, was used as a test sample. The experimental protocol, 100 µg/mL procyanidin B2 solution 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.

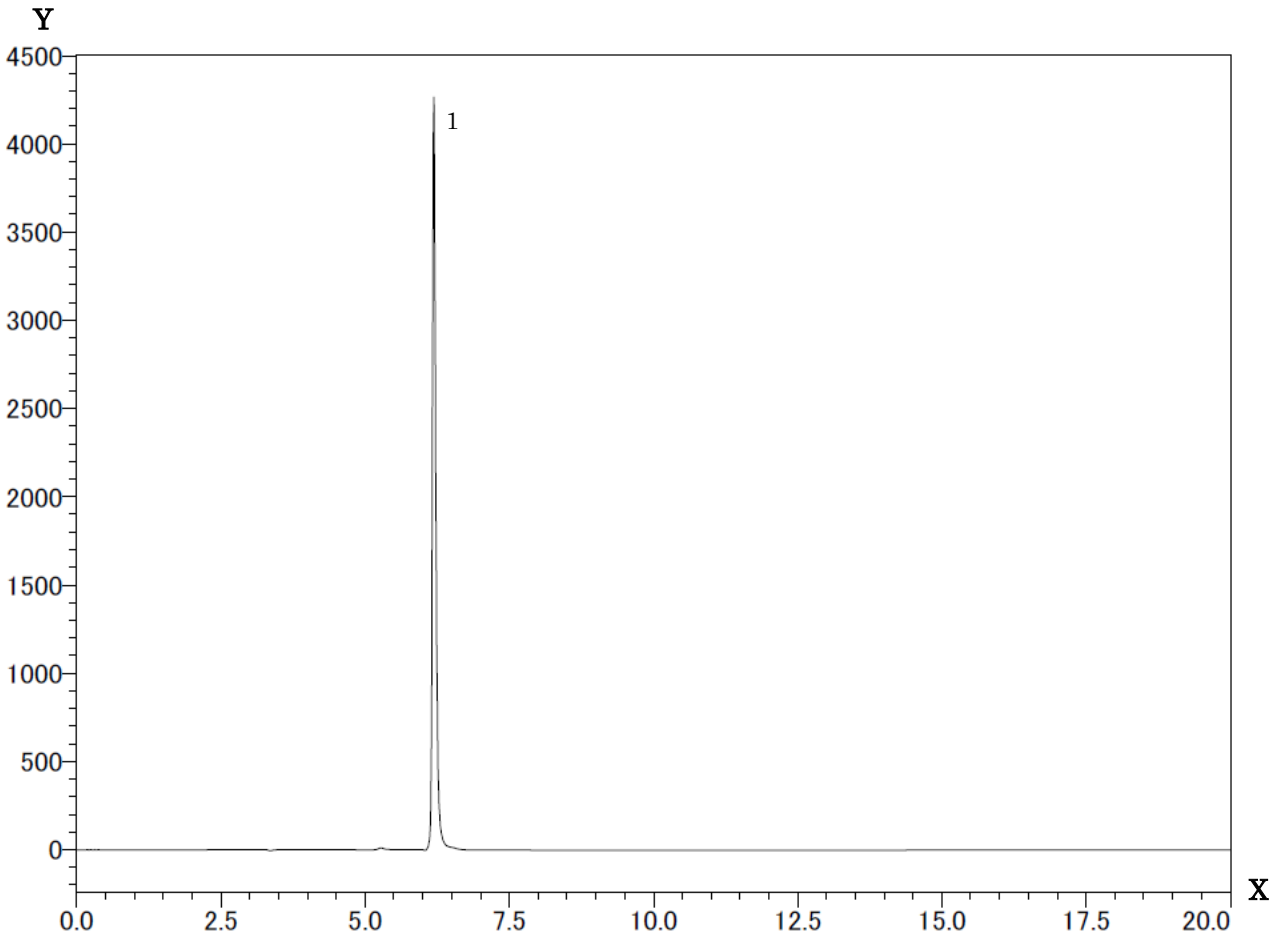
Table A.1 — Precision data

Sample identification	Sample 1	Sample 2	Sample 3	Sample 4
Number of participating laboratories	11	11	11	11
Number of accepted test results	9	9	10	10
Mean procyanidins content, mg/L	88,9	171,4	193,9	253,3
Repeatability standard deviation s_r, mg/L	2,6	2,9	2,1	3,4
Repeatability relative standard deviation RSD_r, %	2,9	1,7	1,1	1,3
Repeatability limit r ($r = 2{,}8\ s_r$), mg/L	7,2	8,1	5,8	9,5
Reproducibility standard deviation s_R, mg/L	8,9	21,3	23,6	31,5
Reproducibility relative standard deviation RSD_R, %	10,0	12,4	12,2	12,4
Reproducibility limit R ($R = 2{,}8\ s_R$), mg/L	24,9	59,7	66,0	88,1

NOTE Although 5 pairs of test samples were supplied according to IUPAC protocol in the interlaboratory tests, it was found that one pair of the sample was short of the specified volume in some laboratories. The data of this sample was not adopted for Table A.1 because participating laboratories were not able to test these samples according to the specified procedure.

Annex B
(informative)

Typical HPLC chromatograms



Key

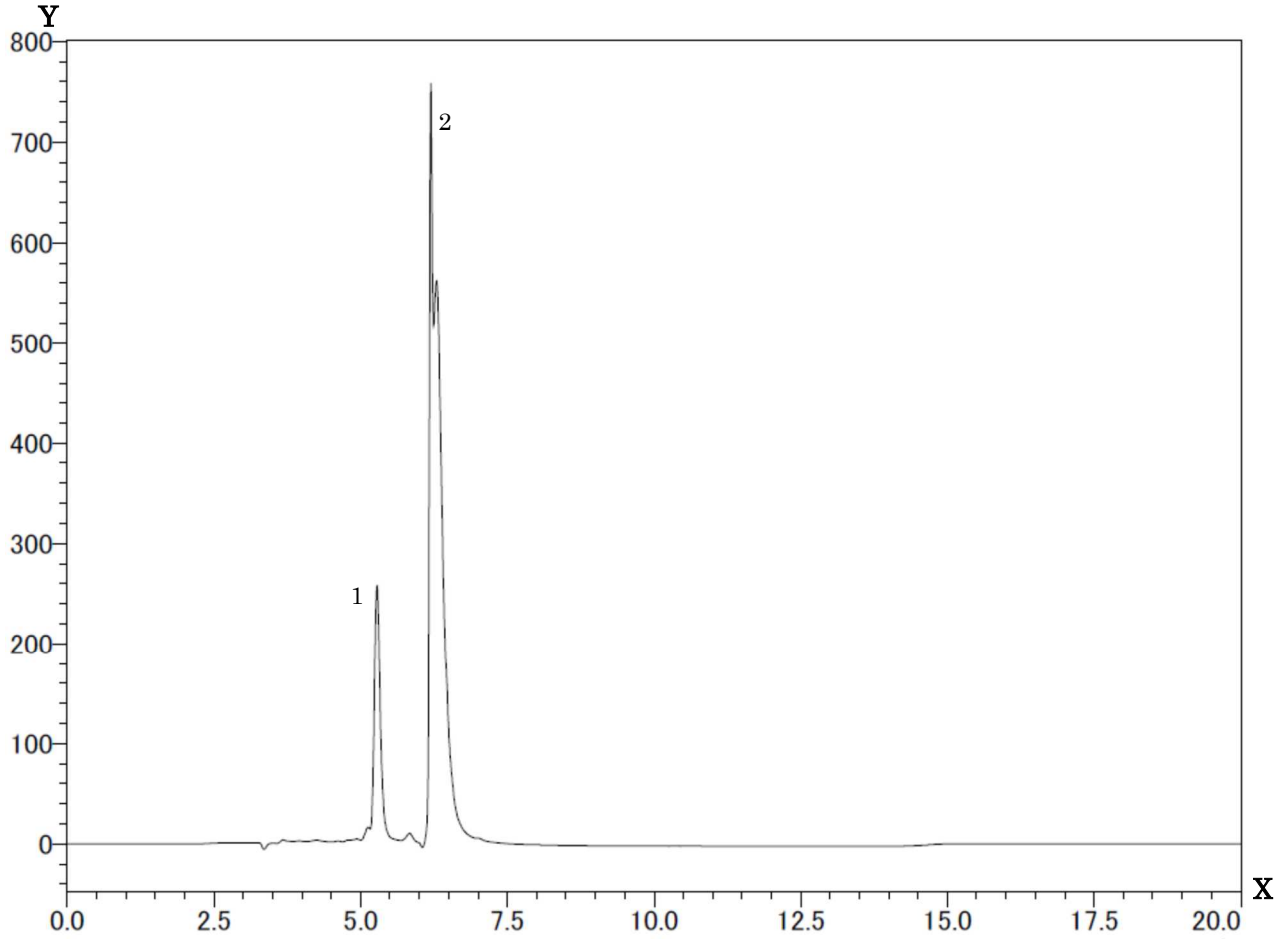
X Retention time, min

Y Response, mV

1 Procyanidin B2

NOTE HPLC system conditions were in accordance with Table 1 and Inertsil® WP300 Diol was used as the column. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

Figure B.1 — 100 µg/mL standard solution



Key

X Retention time, min

Y Response, mV

1 Catechin or epicatechin

2 Procyanidins

NOTE HPLC system conditions were in accordance with Table 1 and Inertsil® WP300 Diol was used as the column. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

Figure B.2 — Apple juice extract

Bibliography

[1] 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.

[2] 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.

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

[4] 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.