Tentative Translation

JAS 0002

JAPANESE AGRICULTURAL STANDARD

Determination of the O-methylated Catechin in ‘Benifuuki’ Green Tea (*Camellia sinensis* L.) —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 revised 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 revision 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 as applied mutatis mutandis pursuant to Article 5 of the same Act. This edition replaces the previous edition (JAS 0002:2019), which has been technically revised.

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JAPANESE AGRICULTURAL STANDARD JAS
(Tentative Translation) 0002 : 2023

Determination of the O-methylated Catechin in 'Benifuuki' Green Tea (*Camellia sinensis* L.) —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 (–)-epigallocatechin 3-(3"-O-methyl)gallate (EGCG3"Me), which is the one of methylated catechins, in tea leaves of only 'Benifuuki' (*Camellia sinensis* var. *sinensis* cv. *Benifuuki*) green tea and those powder products.

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*

ISO 3310-1, *Test sieves — Technical requirements and testing — Part 1: Test sieves of metal wire cloth*

ISO 8655-2, *Piston-operated volumetric apparatus — Part 2: Pipettes*

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

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

JIS K 8101, *Ethanol (99,5) (Reagent)*

JIS K 8107, *Disodium dihydrogen ethylenediamine tetraacetic acid dihydrate (Reagent)*

JIS K 9005, *Phosphoric acid (Reagent)*

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

JIS P 3801, *Filter paper (for chemical analysis)*

3 Terms and definitions

No terms and definitions are listed in this document.

4 Principle

EGCG3"Me is extracted from ground tea leaves using phosphoric acid/ethanol extraction solvent at 30 °C. The extract is diluted with water and the EGCG3"Me in the extract dilution is determined by high performance liquid chromatograph (HPLC) system with UV-visible absorbance detector using gradient elution.

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 EGCG3"Me, of minimum mass fraction, 99 % (HPLC).

5.3 Phosphoric acid, of minimum mass fraction, 85 %, according to JIS K 9005.

5.4 Ethanol, of minimum mass fraction, 99,5 %, according to JIS K 8101.

5.5 Methanol, HPLC grade.

5.6 Acetonitrile, HPLC grade.

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

5.8 Disodium dihydrogen ethylenediamine tetraacetic acid dihydrate, EDTA2Na, of minimum mass fraction, 99,5 %, according to JIS K 8107.

5.9 'Yabukita' green tea, tea leaves or powder products.

5.10 Phosphoric acid solution, mix 49 parts per volume of water with 1 part per volume of phosphoric acid.

5.11 Extraction solvent, mix equal volume of phosphoric acid solution and ethanol.

5.12 Dilution solvent, dissolve 1,76 g of ascorbic acid and 1,00 g of EDTA2Na per 1 000 ml of water.

5.13 Blank extract solution, obtain the extract from 'Yabukita' green tea in accordance with Clause 7 and 8.1. Measure a part of the extract in accordance with 8.3 to obtain the chromatogram. Check this chromatogram, and use an extract whose EGCG3"Me content is confirmed to be below the detection limit as the blank extract solution. Transfer the blank extract solution into deactivated amber vials and store –25 °C or below. Return a blank extract solution to room temperature and mix well before use. Any remaining blank extract solution in the vial shall not be stored again.

NOTE 1 The detection limit can be obtained according to JIS K 0124.

NOTE 2 It has been confirmed that the blank extract solutions remain stable for at least 3 months when stored frozen at –25 °C or below.

5.14 HPLC mobile phases

5.14.1 Mobile phase A, mix 420 parts per volume of water with 1 part per volume of phosphoric acid. Degas the mixture before use.

NOTE Appropriately degassing reduces the influence of dissolved gases in the mobile phase to HPLC determination.

5.14.2 Mobile phase B, mix 18 parts per volume of methanol with 5 parts per volume of acetonitrile. Degas the mixture before use.

NOTE Appropriately degassing reduces the influence of dissolved gases in the mobile phase to HPLC determination.

5.15 EGCG3"Me stock standard solution, dissolve EGCG3"Me in a dilution solvent to prepare a stock

standard solution with an EGCG3"Me concentration of approximately 100 µg/ml. Ultrasound may be used as a means of dissolution. Immediately use this solution to prepare a series of standard solutions (see 5.16), or transfer into deactivated amber vials and store –25 °C or below. If the EGCG3"Me stock standard solution is stored –25 °C or below, return it to room temperature and mix well before use. Do not store any left over stock standard solution after the series of standard solutions have been prepared.

NOTE 1 Because EGCG3"Me in solutions are susceptible to degradation, it is important to add the EDTA2Na and store into deactivated amber vials.

NOTE 2 It has been confirmed that the stock standard solutions remain stable for at least 2 months when stored frozen at –25 °C or below.

5.16 A series of standard solutions, prepare a series of standard solutions from the same EGCG3"Me stock standard solution, and perform the measurement in 8.3.2 on the same day. Mix the EGCG3"Me stock standard solution, dilution solvent and blank extract solution together, and then use to prepare solutions of more than 5 concentration levels that are suited for the measurement of sample extract dilutions. Keep each standard solution in a separate deactivated amber vial. Calculate the actual EGCG3"Me concentration of each standard solution.

NOTE 1 EGCG3"Me is thought to stick to the metal part of the HPLC flow paths, and it is important to add some blank extract solution to the series of standard solutions to mitigate this.

NOTE 2 An example of the prepared series of standard solutions is given in Table 1.

NOTE 3 Between 1 µg/ml and 50 µg/ml of the calibration curve, it has been confirmed that the coefficient of determination is more than 0,990 and the origin is within the 95 % confidence interval of the y-intercept

Table 1 — An example of the prepared series of standard solutions

Standard solutions	Volume taken from stock standard solution, µl	Volume taken from dilution solvent, µl	Volume taken from blank extract solution, µl	The concentration of EGCG3"Me in standard solutions, µg/ml
A	200	700	100	20
B	150	750	100	15
C	100	800	100	10
D	50	850	100	5
E	10	890	100	1

6 Apparatus

The usual laboratory apparatus and the following shall be used.

6.1 Electronic analytical balances, capable of weighing to an accuracy of ±1 mg and capable of weighing to an accuracy of ±0,01 mg.

6.2 Sieve, of nominal size of the aperture 355 µm, of ISO 3310-1.

6.3 One-mark volumetric flasks, to cover the volume range for preparation of EGCG3"Me stock standard solution (see 5.15), extraction (see 8.1) and dilution (see 8.2), of ISO 1042, class A.

6.4 Water bath, capable of being maintained at (30±3) °C.

6.5 Filter papers, conforming to the qualitative analysis filter paper No. 2 of JIS P 3801, and of a size suitable for extraction (see 8.1).

6.6 Membrane filters, hydrophilic PTFE, with a pore size of 0,45 µm.

6.7 Single volume pipettes, to cover the volume range for dilution (see 8.2), of ISO 648, class A.

6.8 Piston pipettes, to cover the volume range for dilution (see 8.2), of ISO 8655-2, type A.

6.9 Deactivated amber vials, made of amber glass, deactivated, to cover the volume range for storage of blank extract solution (see 5.13), storage of EGCG3"Me stock standard solution (see 5.15), determination (see 8.3). The septum of the cap shall be made of PTFE or have PTFE coating.

6.10 HPLC system

6.10.1 HPLC, equipped with a mobile phase delivery system able to perform binary gradient elution, a column oven with temperature control function, an auto sampler, a UV-visible absorbance detector able to measure absorbance at 272 nm and a data processing unit, prescribed in JIS K 0124. A mobile phase delivery system should have a degassing device.

6.10.2 Chromatographic column for HPLC, reverse-phase C18 (ODS) columns, with the following characteristics:

—length: 150 mm;

—internal diameter: 4,6 mm;

—spherical particle size: 5 μm.

—EGCG3"Me shall be eluted with no influence of other components within 12 min. Confirm retention time of EGCG3"Me in accordance with 8.3.

If a guard column is used, select the guard column matching to the C18 (ODS) column.

7 Preparation of test samples

Grind the tea leaves using an appropriate equipment. Sieve the ground samples or powder products to obtain the test sample. Immediately perform the procedure in 8.1, or store the test sample –25 °C or below. If it is stored –25 °C or below, return it to room temperature and mix well before extraction (see 8.1).

NOTE It has been confirmed that the test samples remain stable for at least 2 months when stored frozen at –25 °C or below.

8 Procedure

8.1 Extraction

Weigh, to the nearest 1 mg, 240 mg to 260 mg of the test sample (See Clause 7) into a 25 ml one-mark volumetric flask. Add 20 ml of extraction solvent, and mix slightly. Place the volumetric flask containing the sample in the water bath set at 30 °C, and allow 60 min for the extraction mixture to equilibrate. Remove the volumetric flask from the water bath, and allow it to cool to room temperature. Add water up to the mark. Shake well, and use as blended material.

Filter the blended material using filter paper (discard the first filtrate). Pass the filtrate with a membrane filter (discard the first filtrate), collect approximately 1,5 ml, and use this as the sample extract. Immediately perform the procedure in 8.2 or store the sample extract at–25 °C or below. If it is stored –25 °C or below, return it to room temperature and mix well before dilution.

NOTE 1 In the interlaboratory test described in Annex A, approximately 2 ml of the first filtrate passed through a filter paper was discarded. Furthermore, approximately 1ml of the first filtrate pass through a membrane filter was discarded.

NOTE 2 It has been confirmed that the sample extracts remain stable for at least 2 months when stored frozen at –25 °C or below.

8.2 Dilution

Using a single-volume pipette or a piston pipette, dilute the sample extract (see 8.1) with water, and use this as the sample extract dilution. The dilution may be done with a volumetric flask. It is recommended to mix 1 part per volume of the sample extract with 9 parts per volume of water. Transfer the sample extract dilution into a deactivated amber vial. Immediately perform the procedure in 8.3.

8.3 Determination

8.3.1 HPLC operating conditions

Set up the HPLC system in accordance with the manufacturer's instructions and adjust it as follows.

a) Flow rate of the mobile phase: 1,0 ml/min.

b) Column temperature: 40 °C.

c) Detection wavelength: 272 nm.

d) Volume injected: 10 µl.

e) Binary gradient conditions: 77 % mobile phase A and 23 % mobile phase B for 12 min, then increase the volume ratio of mobile phase B to elute quickly other retained substances from the column. Then reset to 77 % mobile phase A and 23 % mobile phase B, and allow to equilibrate for 10 min before next injection.

NOTE In the interlaboratory test described in Annex A, the binary gradient conditions described in Table 2 were used.

Table 2 — Binary gradient conditions

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0 to 12	77	23
12 to 20	30	70
20 to 30	77	23
NOTE The values given are examples.		

8.3.2 HPLC analysis

Allow the entire system to run for a while to stabilize it. Confirm that the fluctuation of the base line gives no hindrance for the determination of EGCG3"Me by a blank run under the specified conditions (see 8.3.1). Inject the standard solution with the highest concentration (e.g. standard solution A in Table 1) in a series of standard solutions into the column, and compare the chromatogram with that of the blank extract solution to confirm that there are no peaks interfering with the determination of EGCG3"Me in the obtained chromatogram. Then inject the series of standard solutions into the column, followed by the sample extract dilutions (see 8.2). It is recommended to inject a standard solution (e.g. standard solution C in Table 1) at regular intervals (typically after every 5 test solutions).

Collect data using the data processing unit, for all peaks in the standard solutions and the sample extract dilutions.

NOTE The ratio of the largest peak area to the smallest peak area from the peak areas of the standard solution injected at regular intervals is normally 11/9 or less.

8.4 Identification

Identify the individual EGCG3"Me peak in the sample chromatogram by comparing retention times with those obtained from the standard solutions under the same chromatographic conditions (see 8.3.1).

NOTE Typical HPLC chromatograms of a ‘Yabukita’ green tea and a ‘Benifuuki’ green tea are 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. For the peaks of impurities, take appropriate measures according to the perpendicular or tangent method prescribed in JIS K 0124.

9.2 Quantitation

Obtain the EGCG3"Me peak areas for each of the series of standard solutions. Perform a linear regression of peak areas for each standard solution against the EGCG3"Me concentrations of the respective standard solutions to create a calibration curve.

Calculate the concentration of EGCG3"Me in each sample extract dilution by using the calibration curve. The EGCG3"Me content, w_C, is given by the formula:

$$w_C = \frac{C \times V \times d \times 1\,000}{m \times 1\,000}$$

where

w_C is the EGCG3"Me content in the test sample (g/kg);

C is the concentration of EGCG3"Me in the sample extract dilution (μg/ml);

V is the volume (ml) of extraction solvent, typically 25 (see 8.1);

d is the dilution factor for the sample extract dilution, typically 10 (see 8.2);

m is the mass (mg) of the test sample.

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 (11 g/kg to 19 g/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 [1] given in Table A.1 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 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 test

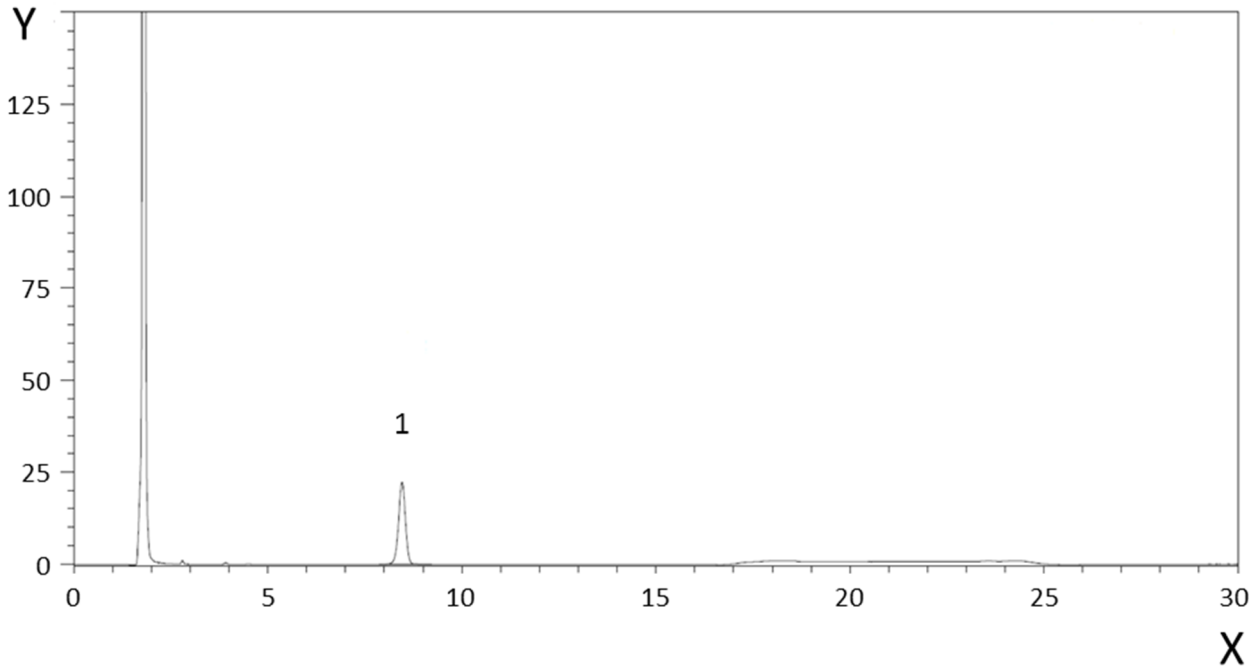
An interlaboratory test was carried out in accordance with IUPAC protocol [3] in 2015 in Japan, and gave the statistical results given in Table A.1 [4]. The homogenous [5] test samples were prepared from commercially available tea leaves and powder products of 'Benifuuki' green tea. The experimental protocol, test samples, an EGCG3"Me stock standard solution with known concentration and a blank extract solution 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 10 test samples (5 pairs of blind duplicates) according to the experimental protocol.

Table A.1 — Precision data

Sample identification	**Sample 1 (tea leaves)**	**Sample 2 (powder product)**	**Sample 3 (powder product)**	**Sample 4 (powder product)**	**Sample 5 (powder product)**
Number of participating laboratories	10	10	10	10	10
Number of accepted test results	10	8	10	8	10
Mean EGCG3"Me content, g/kg	10,85	10,77	13,65	15,59	18,89
Repeatability standard deviation s_r, g/kg	0,22	0,15	0,20	0,21	0,30
Repeatability relative standard deviation RSD_r, %	2,0	1,4	1,5	1,4	1,6
Repeatability limit r ($r = 2{,}8\ s_r$) , g/kg	0,61	0,42	0,57	0,59	0,83
Reproducibility standard deviation s_R, g/kg	0,62	0,17	0,54	0,25	0,76
Reproducibility relative standard deviation RSD_R, %	5,7	1,6	4,0	1,6	4,0
Reproducibility limit R ($R = 2{,}8\ s_R$), g/kg	1,7	0,47	1,5	0,71	2,1

Annex B
(informative)

Typical HPLC chromatograms



Key

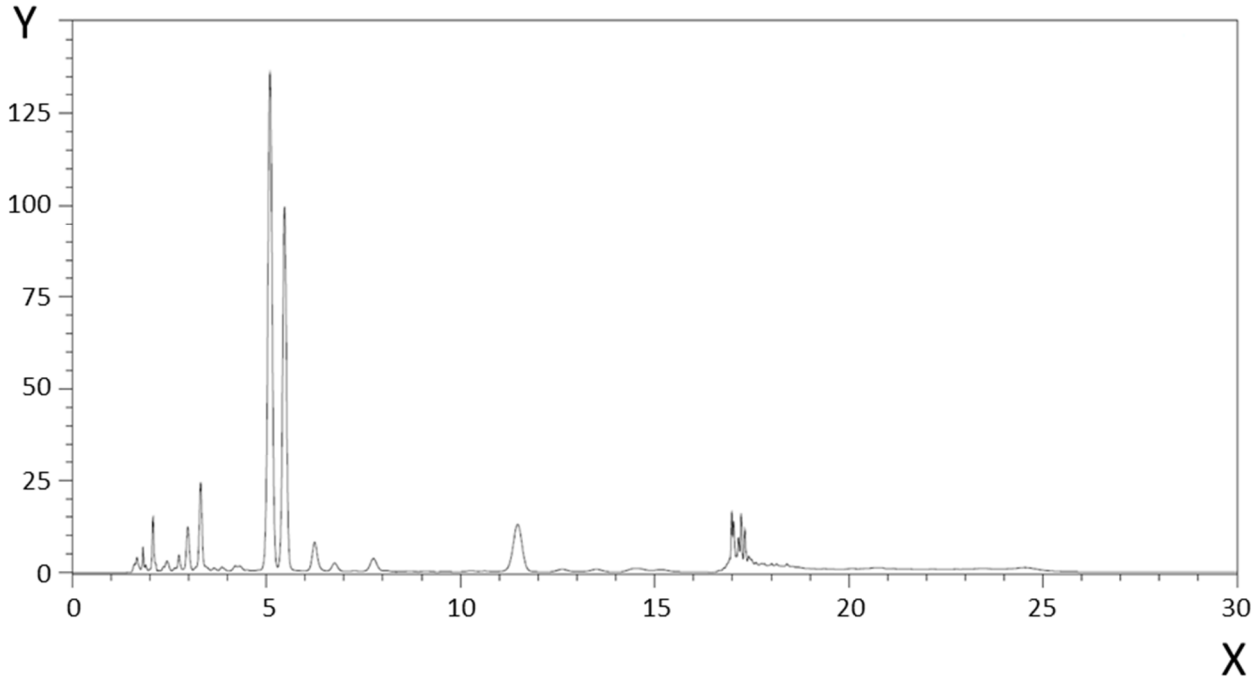
X retention time, min

Y response, mAU

1 EGCG3"Me

NOTE For the HPLC operating conditions, in addition to 8.3.1, a binary gradient conditions of Table 2 was used, and Wakopak® Navi C18-5 was used as the column. This information is given for the convenience of the users of this document and does not constitute an endorsement of the product.

Figure B.1 — EGCG3"Me standard solution (does not contain blank extract solution)



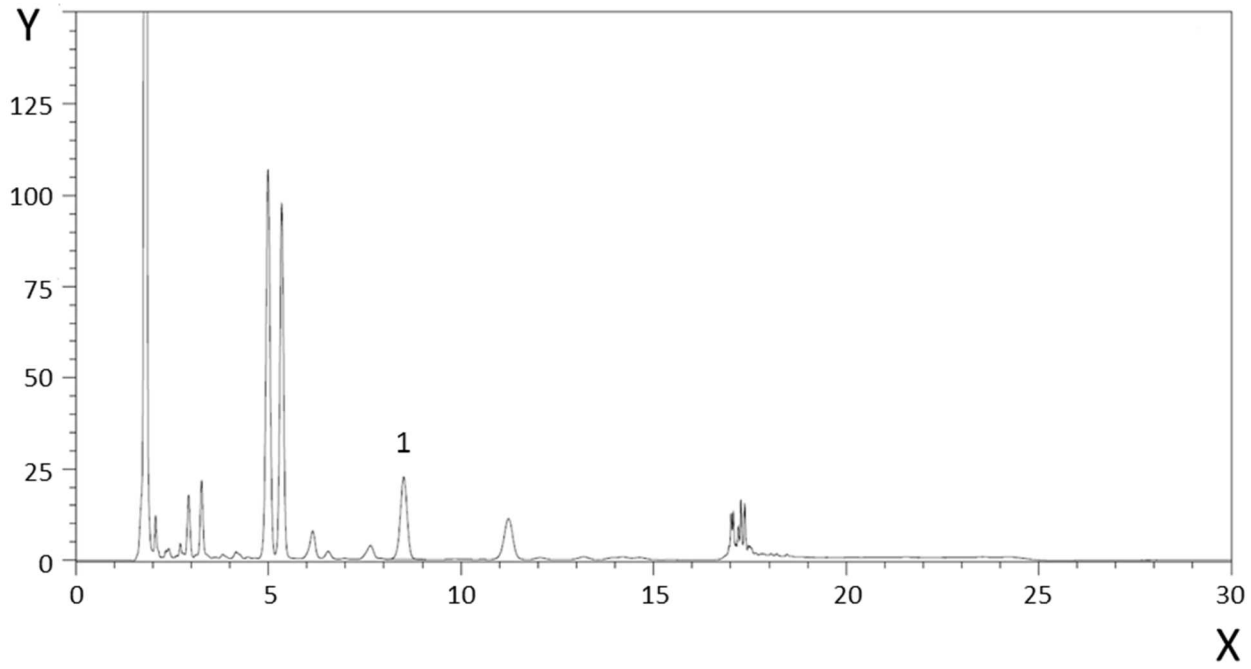
Key

X retention time, min

Y response, mAU

NOTE For the HPLC operating conditions, in addition to 8.3.1, a binary gradient conditions of Table 2 was used, and Wakopak® Navi C18-5 was used as the column. This information is given for the convenience of the users of this document and does not constitute an endorsement of the product.

Figure B.2 — Blank extract solution



Key

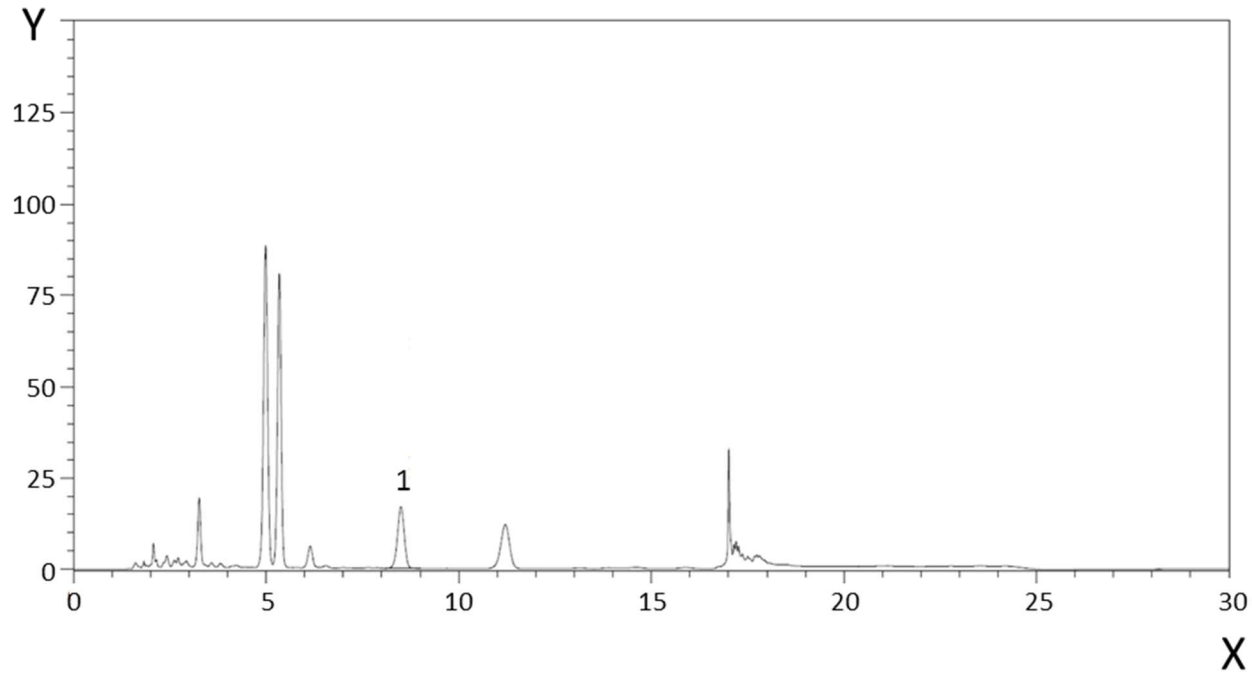
X retention time, min

Y response, mAU

1 EGCG3"Me

NOTE For the HPLC operating conditions, in addition to 8.3.1, a binary gradient conditions of Table 2 was used, and Wakopak® Navi C18-5 was used as the column. This information is given for the convenience of the users of this document and does not constitute an endorsement of the product.

Figure B.3 — EGCG3"Me standard solution



Key

X retention time, min

Y response, mAU

1 EGCG3"Me

NOTE For the HPLC operating conditions, in addition to 8.3.1, a binary gradient conditions of Table 2 was used, and Wakopak® Navi C18-5 was used as the column. This information is given for the convenience of the users of this document and does not constitute an endorsement of the product.

Figure B.4 — Sample extract dilution

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] Homura, Y., et al., Validation of Method for Determining *O*-methylated Catechin in 'Benifuuki' Green Tea (*Camellia sinensis* L.) by Interlaboratory Study, *Nippon Shokuhin Kagaku Kogaku Kaishi*, 2016, **63**(7), pp. 312-318.

[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.